Mass Spectrometry Measurement of Metals and Metalloproteins in Brain Tissue.

A Thesis Presented for the Degree of Doctor of Philosophy

by

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at

The University of Newcastle

School of Environmental and Life Sciences

Callaghan NSW 2308

December 2011
Statement of Originality

The thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. I give consent to this copy of my thesis, when deposited in the University Library, being made available for loan and photocopying subject to the provisions of the Copyright Act 1968.

Mitchell Paul
Dedication

To my wife Saina for her faith and support throughout the production of this thesis.
Acknowledgements

I would like to express my gratitude to my supervisors Professor Mike Calford, Associate Professor Hugh Dunstan and Dr. Carl Parsons for their excellent advice and support during the course of this project. In particular I appreciate their patience for what ended up being a particularly longer project than usual. In addition, I would like to thank Dr. Mark Graham and the Children’s Medical Research Institute for generous use of their facility’s resources and time.
Abstract

Metal ions carry out a number of essential processes in physiological functions including neuronal signalling, gene expression and catalysis. Increasing attention towards the roles that metals play has been largely driven by their implication in neurological disorders such as ALS, AD and PD. While systematic studies have been carried out on the CNS in the diseased state few have been performed on the same healthy tissues in a standardized fashion. Furthermore, there is a paucity of quantitative data concerning metal distribution during neural re-organization and plasticity.

The normal distribution of a range of elements in the brain tissue of healthy Wistar rats was established using Inductively Coupled Plasmas Sector Field Mass Spectrometry (ICP-SF-MS). A method was developed to determine concentrations of Ag, Cd, Hg, Pb, Bi, U, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As and Se in specific brain regions. A principal component analysis was then used to reveal elemental patterns of the brain regions. This standardized method was used in subsequent investigations of the barrel cortex thereby allowing direct comparisons to be made given that the sampling, sample preparation and instrument operation were performed under the same analytical conditions. In contrast, most “reference values” for trace element content in the CNS of “normal” populations gather results from disparate studies performed by researchers using distinct techniques and a wide variety of instruments.

The fluctuation of Zn was investigated in the barrel cortex of rats contralateral and ipsilateral to partial vibrissae removal (checkerboard pattern plucking). Quantitative analysis was carried out using ICP-SF-MS. This allowed measurement of total Zn content and separated low molecular weight (free) and protein bound components. A significant decrease of Zn at 8 h after whisker plucking was revealed in both deprived
and non-deprived barrel cortices. Thereafter, zinc content increased above control levels for the length of the study (up to 96 h), confirming previous studies using histochemical staining methods for free zinc. All changes are attributable to free Zn as no change was observed in the protein bound form as sampled at 8 and 24 hr post manipulation.

From the same study additional data is presented on the fluctuation of metalloproteins and unbound metals in the barrel cortex after vibrissae removal. Copper, Mn, Fe and Mg fluctuation were investigated in the barrel cortex of rats with subsequent analysis using ICP-SF-MS and Electrospray Ionization Time-of-Flight Mass Spectrometry (ESI-TOF-MS) for metal quantification and protein identification, respectively. A significant decrease of Mn and Cu at 8 h after whisker plucking was revealed in both deprived and non-deprived barrel cortices. Changes to Cu were attributable to protein-bound species and changes to Mn were attributed to both protein-bound and non-protein bound species. The significant decrease of the metals Cu and Mn (along with Zn) at 8 h post-vibrissectomy indicated a synchronised compensatory mechanism of the CNS for reduced sensory input given their role in modulation of neural activity. In addition, several proteins of interest were identified for further investigation demonstrating the utility of ICP-SF-MS as a screening technique for metalloproteins. Coupled with high resolution separation of the protein, ICP-SF-MS offers itself as an innovative analytical tool. Given that approximately a third of proteins require a metal cofactor, a systematic approach to the study of metal content, speciation, localization and use within the CNS is supported.
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Journal Articles


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<td>AAS</td>
<td>Atomic Absorption Spectroscopy</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s Disease</td>
</tr>
<tr>
<td>AEC</td>
<td>Anion Exchange Chromatography</td>
</tr>
<tr>
<td>ALS</td>
<td>Amyotrophic Lateral Sclerosis</td>
</tr>
<tr>
<td>AMPB</td>
<td>Aminopeptidase B</td>
</tr>
<tr>
<td>AMPL</td>
<td>Cytosol Aminopeptidase</td>
</tr>
<tr>
<td>CE</td>
<td>Capillary Electrophoresis</td>
</tr>
<tr>
<td>CEC</td>
<td>Cation Exchange Chromatography</td>
</tr>
<tr>
<td>CNDP2</td>
<td>Cytosolic non-specific Dipeptidase</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>CRM</td>
<td>Certified Reference Material</td>
</tr>
<tr>
<td>DAC</td>
<td>Digital-to-Analog Converter</td>
</tr>
<tr>
<td>DAD</td>
<td>Diode Array Detector</td>
</tr>
<tr>
<td>DEAE</td>
<td>DiEthylAminoEthane</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>ESA</td>
<td>Electric Sector Analyzer</td>
</tr>
<tr>
<td>ESI-MS</td>
<td>Electrospray Ionization Mass Spectrometry</td>
</tr>
<tr>
<td>ESI-Q-TOF</td>
<td>ESI Quadrupole Time-of-Flight</td>
</tr>
<tr>
<td>GLNA</td>
<td>Glutamine synthetase</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Pressure Liquid Chromatography</td>
</tr>
<tr>
<td>HRM</td>
<td>High Resolution Mode</td>
</tr>
<tr>
<td>IDHC</td>
<td>Isocitrate Dehydrogenase (NADP) Cytoplasmic</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>ICP</td>
<td>Inductively Coupled Plasma</td>
</tr>
<tr>
<td>ICP-AES</td>
<td>ICP Atomic Emission Spectroscopy</td>
</tr>
<tr>
<td>ICP-MS</td>
<td>ICP Mass Spectrometry</td>
</tr>
<tr>
<td>ICP-Q-MS</td>
<td>ICP Quadrupole Mass Spectrometry</td>
</tr>
<tr>
<td>ICP-SF-MS</td>
<td>ICP Sector Field Mass Spectrometry</td>
</tr>
<tr>
<td>IEC</td>
<td>Ion Exchange Chromatography</td>
</tr>
<tr>
<td>LA</td>
<td>Laser Ablation</td>
</tr>
<tr>
<td>LOD</td>
<td>Limits of Detection</td>
</tr>
<tr>
<td>LRM</td>
<td>Low Resolution Mode</td>
</tr>
<tr>
<td>MALDI</td>
<td>Matrix Assisted Laser Desorption Ionization</td>
</tr>
<tr>
<td>MAOX</td>
<td>NADP-dependent malic enzyme</td>
</tr>
<tr>
<td>MDAC</td>
<td>Mass Digital-to-Analog Converter</td>
</tr>
<tr>
<td>MDLC</td>
<td>Multi-dimensional Liquid Chromatography</td>
</tr>
<tr>
<td>MRM</td>
<td>Medium Resolution Mode</td>
</tr>
<tr>
<td>MT</td>
<td>Metallothionein</td>
</tr>
<tr>
<td>MWCO</td>
<td>Molecular Weight Cut-Off</td>
</tr>
<tr>
<td>NAA</td>
<td>Neutron Activation Analysis</td>
</tr>
<tr>
<td>PCA</td>
<td>Principal Component Analysis</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s Disease</td>
</tr>
<tr>
<td>PEEK</td>
<td>Polyether Ether Ketone</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
<td>---------</td>
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<tr>
<td>PET</td>
<td>Positron Emission Tomography</td>
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<tr>
<td>PIXES</td>
<td>Proton Induced X-Ray Emission Spectroscopy</td>
</tr>
<tr>
<td>PP1A</td>
<td>Serine/threonine-protein phosphatase PP1-alpha catalytic subunit</td>
</tr>
<tr>
<td>PP1B</td>
<td>Serine/threonine-protein phosphatase PP1-beta catalytic subunit</td>
</tr>
<tr>
<td>PP1G</td>
<td>Serine/threonine-protein phosphatase PP1-gamma catalytic subunit</td>
</tr>
<tr>
<td>PPM</td>
<td>Parts per million</td>
</tr>
<tr>
<td>PPP5</td>
<td>Serine/threonine-protein phosphatase</td>
</tr>
<tr>
<td>PPT</td>
<td>Parts per trillion</td>
</tr>
<tr>
<td>RF</td>
<td>Radio Frequency</td>
</tr>
<tr>
<td>RPLC</td>
<td>Reversed Phase Liquid Chromatography</td>
</tr>
<tr>
<td>SEC</td>
<td>Size Exclusion Chromatography</td>
</tr>
<tr>
<td>SEM</td>
<td>Secondary Electron Multiplier</td>
</tr>
<tr>
<td>SODC</td>
<td>Superoxide Dismutase (Cu/Zn)</td>
</tr>
<tr>
<td>SODM</td>
<td>Superoxide Dismutase (Mn)</td>
</tr>
<tr>
<td>SRM</td>
<td>Standard Reference Material</td>
</tr>
<tr>
<td>TFM</td>
<td>Tetrafluoromethane</td>
</tr>
<tr>
<td>UHV</td>
<td>Ultra High Vacuum</td>
</tr>
<tr>
<td>XPP1</td>
<td>Xaa-Pro aminopeptidase 1</td>
</tr>
<tr>
<td>XRF</td>
<td>X-Ray Fluorescence</td>
</tr>
</tbody>
</table>
Chapter One: Probing Metalloneurochemistry Using Physical Methods

1.1 Introduction

1.1.1 A definition of metalloneurochemistry

Metalloneurochemistry was coined to describe the study of metal ions in the CNS at a molecular level (Burdette and Lippard 2003). More recently, the term metalloneurobiology (Bush and Curtain 2008) has been introduced into the neuroscience lexicon, although this field focuses on metal species causative of neurodegenerative diseases (in particular Cu and Zn) (Lippard and Berg 2000). Historically neurochemists and neurophysiologists undertook these investigations.

With the discovery that metal ions perform structural and catalytic functions in proteins and enzymes (Lippard and Berg 2000), a new interface between chemistry and biology was created. Before its inception, the primary focus of biochemists was on biomolecules, such as proteins and DNA, with the metal ions drawing the least attention (Bush 2000; Burdette and Lippard 2003). On the other hand, inorganic chemists were more prone to concentrate exclusively on metal coordination chemistry, therefore overlooking the importance of macro-molecular structures and so vacating this sub-discipline to the bioinorganic/organometallic chemists (Burdette and Lippard 2003). Notwithstanding the latter contribution, advances in understanding the role of metal ions in biological systems depended largely on breakthroughs in the development of analytical methods. Figure 1 shows the inter-relationships between the various sub-disciplines to metalloneurochemistry thereby highlighting that metalloneurochemistry is an integrating science, representing the molecular and mechanistic areas of the neurosciences. As
pointed out by Ashley Bush (Bush 2000), fundamental to an appreciation of these interfaces “is an awareness that the brain is a specialized organ that concentrates metal ions”.

![Diagram showing the inter-relationship between metalloneurochemistry and the other science disciplines.]

Figure 1.1 The inter-relationship between metalloneurochemistry and the other science disciplines.

The earliest investigations in metalloneurochemistry centered on the Na and K ions in neurotransmission. It was shown that these ions participated in the most important and fundamental function of the CNS; that is, they regulated the opening and closing of ion channels that mediated their passage through the plasma membranes, creating electrochemical gradients in order to transmit information and regulate cellular function (Devlin 1992). Other studies centered on Ca and Zn ions. The Ca cation was shown to be the most important metal ion in signal transduction with changes in its intracellular concentrations initiating a cascade of signalling events. On the other hand, the importance of Zn was reflected by its high concentrations in the CNS serving as an intercellular signalling messenger that was released from central nerve terminals during synaptic...
activity (Choi and Koh 1998). Zinc has the potential to alter the behaviour of multiple membrane channels and neurotransmitter receptors, although the exact role that neuronal Zn release plays in normal brain functions has not yet been fully established. There is increasing evidence to suggest that synaptic released Zn has a key role in disease pathogenesis (Choi and Koh 1998). In particular, Zn has been implicated as the factor responsible for aggregating β-amyloid (Bush 2003) and other protein aggregates which are associated with neurodegenerative diseases such as AD, PD and ALS (Barnham et al. 2004).

These early investigations were representative of the first category of metal ion environments in the CNS, namely, that of the unbound metal ion. The second category centered on metal ions that were bound (primarily) to proteins, which was recently proposed (in a broader sense) as a new scientific sub-field termed “metallomics” (Haraguchi 2004). Metallomics had evolved out of proteomics and metabolomics with the aim to integrate metal speciation studies within life sciences. Moreover, Maret (Maret 2004) identified the need to classify all metalloproteins and to establish a comprehensive database for the role of essential metals in health and disease, with a focus on Zn proteins, since they formed a major fraction of metalloproteins as unbound Zn only accounted for a small percentage of the total Zn content in the brain. The Zn that is bound to proteins functions either as a component of the catalytic site of enzymes or in a structural capacity (Cuajungco 1997).

The importance of metal speciation was predicted more than two decades ago by analytical electrochemists (Florence 1980) and ever since it has been continuously growing in significance. For example, trace metals play critical roles in the CNS and in order to understand their essential, toxic or therapeutic functions and associations to key biomolecules (e.g. DNA, RNA, polypeptides, proteins) thorough and systematic
investigations need to be undertaken. The next generation instruments in such investigations will center on the ICP-SF-MS since they are the only bio-analytical tools currently available that have the resolution, sensitivity and universality to undertake such tasks.

1.1.2 The need to focus on metalloneurochemistry

Human neurodegenerative diseases are a major health concern, especially as life expectancy increases in the developed societies (Case 2005). Due to the increasing heavy metal pollution experienced in industrial countries, neurological studies of trace metal elements not only yielded knowledge on the appropriateness of the environment for human and animal habitation, but also on possible causes for neurological disorders (Atwood and Yearwood 2000). Hence, interest has increased in the quantification of essential and toxic trace metal elements in neurological tissues, driven largely by the association of neurological disorders with the accumulation of toxic amounts of metal ions in the CNS. In order to diagnose, investigate and treat the conditions caused by these metals, the biological distribution and fate of trace metal elements in the near future will have to be accurately mapped (Chan et al. 1983).

Earlier studies have shown that many trace metal elements were carefully balanced and played a pivotal metabolic role (Cumings 1959; Cumings 1965; Kozma and Ferke 1970; Cooper et al. 1978; Rath et al. 1980; Usdin et al. 1980). If these balances were actively disturbed via exposure routes, such as from the ingestion or inhalation of high dosages over extended periods of time, they may become toxic. For example, studies on postmortem brains from PD patients revealed elevated Fe levels in the substantia nigra. Selective cell death in this brain region is associated with oxidative stress, which may be exacerbated by the presence of excess Fe (Kaur et al. 2003).
Another study had discovered significant changes of Fe and Zn concentrations in some AD brain regions compared to those from normal brain tissue (Andrasi et al. 2000). Srivastava and Jain (Srivastava and Jain 2002) showed that the increased concentrations of some metal ions may have cytotoxic effects through an ability to oxidatively modify biomolecules, which may cause oxidative stress-induced brain cell death leading to neurodegenerative disorders observed in AD. Bush and co-workers (Friedlich et al. 2003; Huang et al. 2004) have discovered that the major proteins implicated in AD (i.e. Aβ and the amyloid protein precursor (APP)) are Cu/Zn metalloproteins whose physicochemical properties are profoundly influenced by interaction with these metal ions. Furthermore, their in vitro data was supported by finding that Zn, Cu and Fe were enriched in AD neocortex and more so in the amyloid plaque deposit. Recently, Bush and co-workers (Duce et al. 2010) have found that APP actually displays ferroxidase activity that is inhibited specifically by Zn. Given the range of oxidation states available to transition metals and their consequent ability to impose conformational changes upon the proteins to which they bind, it is not surprising that they have been implicated in protein misfolding (which is a common link between neurodegenerative diseases such as AD, PD and ALS) (Case 2005).

Morawski et al. (Morawski et al. 2005) have investigated the intra- and extra-neuronal elemental concentrations (in particular Fe) of the human substantia nigra pars compacta versus pars reticula, allowing the comparison of neuronal Fe concentrations in human brain sections of healthy and Parkinsonian brain tissue. In addition, these researchers showed, in situ, that the increased intra-neuronal Fe content was linked to neuromelanin. Neuromelanin is a dark polymer pigment produced in specific populations of catecholaminergic neurons in the brain. Interest in neuromelanin was due to the contemplated link between it and the especial vulnerability of neuromelanin-containing
neurons to cell death in PD (Nicolaus 2005). Also Zecca et al. (Zecca et al. 1994) discovered relatively high levels (30,800 µg g\(^{-1}\)) of Fe in neuromelanin compared with other human brain tissues. It was found that neuromelanin has a particular affinity for other metals such as, Zn, Cr, Se, Sr, Co, Sb, Ni, Hg, Ce, Au, Ag, Ta, and Sc.

Most elements have been shown to be essential to life when present at the appropriate concentration (i.e. trace concentration). The maintenance of metal ion homeostasis is an important topic in metalloneurochemistry (Burdette and Lippard 2003). Table 1.1 gives the metal ions, their chemical characteristics and biochemical roles. For other elements, toxicity has been established even when concentrations are low (e.g. Hg). It is therefore not surprising that the determination of trace metal elements is of paramount importance in both clinical and biomedical research (Moens 1997).

In recent years, interest has increased in the determination of essential and toxic trace metal elements in neurological tissues. This has been driven largely by the association of neurological disorders with the accumulation of toxic amounts of metal ions in the CNS. In order to recognize, study and treat the conditions caused by these metals, the biological distribution and fate of trace metal elements must be better understood (Chan et al. 1983). Human neurodegenerative diseases are a major health concern to society as a whole, as they are becoming increasingly common as life expectancy increases (Case 2005). For example, AD is the most prevalent form of dementia and affects more than 37 million people world-wide, with an estimated cost of $422 billion in 2009 (Wimo et al. 2010). It is estimated that by 2050 the number of people over 60 in Europe will have doubled to 40 % of the total population or 60 % of the working age population. With respect to the United States, people aged 65 or older now comprise around 13 % of the population. On a global level, there will be around one billion elderly by the year 2025 with this number doubling by 2050 (Case 2005).
Table 1.1 Chemical characteristics and biochemical roles of various metals and metalloids in the brain (Cotton et al. 1987; Shriver and Atkins 1999; Nelson and Cox 2005).

<table>
<thead>
<tr>
<th>Metal</th>
<th>Chemical characteristics</th>
<th>Biochemical role</th>
</tr>
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<tbody>
<tr>
<td>Na and K</td>
<td>The highly mobile mono-cations form mainly soluble salts but only weak complexes with most ligands with the exception of the cyclic polydentate oxygen donors.</td>
<td>Concentration differences created by active transport provide functional osmotic and electrochemical gradients. These ions may also help stabilize a particular protein conformation by binding at its anionic sites.</td>
</tr>
<tr>
<td>Mg and Ca</td>
<td>The ions Mg(^{2+}) and Ca(^{2+}) are hard Lewis acids and preferentially bind to anionic oxygen donor ligands such as phosphate. None of these metals have any redox chemistry.</td>
<td>These ions act as enzyme activators and Lewis acids or structure promoters. These metals often compete for binding and may inhibit activity at each other’s sites, e.g. NMDA receptor. The Ca concentration in muscle is actively controlled to act as a neuromuscular “trigger”.</td>
</tr>
<tr>
<td>Cr</td>
<td>Complexes for oxidation states from +6 to –2 are known. However, Cr (III) is the principle species. In higher oxidation states its compounds are powerful oxidants. Cr (III) is a hard Lewis acid and binds readily to O and N donor ligands.</td>
<td>Cr (III) forms an essential part of the Glucose Tolerance Factor together with insulin. Cr also affects amino and nucleic acids synthesis.</td>
</tr>
<tr>
<td>Mn</td>
<td>Mn is found in oxidation states ranging from +7 to –3, although Mn (II) is the most stable state in aqueous solutions. The higher oxidation states all have oxo-ligands and are oxidizing agents.</td>
<td>Mn (II) activates several enzymes (often in competition with Mg) including peptidases, phosphatises and polymerases. Mn is an essential cofactor of a few enzymes in carbohydrate biochemistry (e.g. catalase).</td>
</tr>
<tr>
<td>Fe</td>
<td>Fe (II) and (III) are the principal oxidation states. Fe(^{2+}) binds preferentially to nitrogen donor ligands and Fe(^{3+}) to oxygen donor anions.</td>
<td>Fe compounds are of two types: Fe-protein-sulfide and Fe porphyrins, which are the principal electron carriers in biological redox reactions. The functions of Fe include: electron transport; O(_2) transport; catalysis of oxidoreductase reactions. Approximately 75% of Fe in human beings is in the form of the oxygen-carrying pigment, haemoglobin.</td>
</tr>
<tr>
<td>Co</td>
<td>Two oxidation states are common: Cobalt (II) and (III). The former is kinetically labile and the latter inert.</td>
<td>The cobalt-corrin complex and Vitamin B(_{12}), is an important coenzyme, whilst the Co(^{3+}) ion is a cofactor for some hydrolytic enzymes.</td>
</tr>
<tr>
<td>Cu</td>
<td>The most usual oxidation states are copper (I) and (II), which are reversible.</td>
<td>Cu forms a vital part of a number of oxidases and catalytic proteins. These enzymes operate by means of a redox cycle and so have a similar biochemistry to Fe.</td>
</tr>
</tbody>
</table>
Table 1.1 continued

<table>
<thead>
<tr>
<th>Metal</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>Al and Tl</td>
<td>Al is found primarily as +3 complexes and given this high charge forms many insoluble salts. A range of stable Al complexes are known, particularly with anionic oxygen donor ligands. Al is a hard Lewis acid. Tl is a soft Lewis acid and has a moderate affinity for S ligands. Tl may form (I) and (III) ions.</td>
</tr>
<tr>
<td>Zn, Cd and Hg</td>
<td>These metals prefer to form compounds with ligands such as Cl, P or S donor atoms. Complexes with these metals have considerable covalent character since they are soft Lewis acids (with the exception of Zn which is borderline since it will equally bind to N). Cd and Hg are highly toxic. These metals have a strong affinity for thiol groups and therefore their toxic action may be related to the substitution of Zn in an enzyme system. Zn is an essential cofactor in many hydrolase enzymes. Its many functions can be described as structure-promotion or enzyme activation. Zn has no redox chemistry.</td>
</tr>
<tr>
<td>Sn and Pb</td>
<td>Each metal forms typically ionic compounds in the lower oxidation state (II). In the higher oxidation state (IV) they have considerable covalent character, which is reflected by the range of organometallic compounds formed. The toxicity status of Sn is uncertain, although some organo-tin complexes have been shown to be toxic. Pb is extremely toxic.</td>
</tr>
<tr>
<td>As</td>
<td>Common oxidation states are +3 and +5. As forms a variety of inorganic compounds, particularly with O, and a number of organic species. As and its compounds are toxic (in particular the inorganic forms). Its toxicity is due to allosteric inhibition of the metabolic enzyme, lipothiamide pyrophosphatase. Se is toxic in large doses but is an essential micronutrient. It is a component of the amino acids, selenocysteine and selenomethionine. Se forms a part of the antioxidant, glutathione peroxidise. The inorganic forms of Se are highly toxic and has modes of action similar to As.</td>
</tr>
<tr>
<td>Se</td>
<td>Common oxidation states include −2, +4, +6. Se has a similar chemistry to As and forms a number of compounds with halogens, O and S.</td>
</tr>
</tbody>
</table>

1.1.3 Unbound metal and metalloproteins

Throughout biological systems, metallic ions are closely linked with proteins. Although neurotransmitters are essentially organic molecules, metals are involved in
some of the key steps in the process of neurotransmission (Guo et al. 2002). In Table 1.2 are highlighted some metalloproteins found in the CNS.

Table 1.2 Typical metalloproteins and their biological functions (Guo et al. 2002; Haraguchi 2004).

<table>
<thead>
<tr>
<th>Metalloprotein</th>
<th>Metals</th>
<th>Biological function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calmodulin</td>
<td>Ca</td>
<td>Enzyme activation</td>
</tr>
<tr>
<td>Cyanocobalamin</td>
<td>Co</td>
<td>Vitamin B$_{12}$</td>
</tr>
<tr>
<td>Ceruloplasmin</td>
<td>Cu</td>
<td>Oxidation of Fe</td>
</tr>
<tr>
<td>Dopamine β-hydroxylase</td>
<td>Cu</td>
<td>Conversion of dopamine to noradrenaline</td>
</tr>
<tr>
<td>Haemoglobin</td>
<td>Fe</td>
<td>Oxygen carrier</td>
</tr>
<tr>
<td>Myoglobin</td>
<td>Fe</td>
<td>Oxygen carrier</td>
</tr>
<tr>
<td>Transferrin</td>
<td>Fe</td>
<td>Transportation of Fe</td>
</tr>
<tr>
<td>Ferritin</td>
<td>Fe</td>
<td>Storage of Fe</td>
</tr>
<tr>
<td>Tyrosine hydroxylase</td>
<td>Fe</td>
<td>Conversion of L-tyrosine to dopamine</td>
</tr>
<tr>
<td>Catarase</td>
<td>Fe</td>
<td>Decomposition of H$_2$O$_2$</td>
</tr>
<tr>
<td>Cytochrome P-450</td>
<td>Fe</td>
<td>Metabolism of steroids</td>
</tr>
<tr>
<td>ATPase</td>
<td>Mg</td>
<td>Hydrolysis of ATP</td>
</tr>
<tr>
<td>Glutamine synthetase</td>
<td>Mn</td>
<td>Conversion of glutamate to glutamine</td>
</tr>
<tr>
<td>Transmanganin</td>
<td>Mn</td>
<td>Transport of Mn</td>
</tr>
<tr>
<td>Superoxide dismutase</td>
<td>Mn or Zn, Cu</td>
<td>Catalysis of superoxide disproportionation</td>
</tr>
<tr>
<td>Channel protein</td>
<td>Na, K</td>
<td>Gated transport across membrane</td>
</tr>
<tr>
<td>Nickeloplasmin</td>
<td>Ni</td>
<td>Transportation of Ni</td>
</tr>
<tr>
<td>Glutathione peroxidase</td>
<td>Se</td>
<td>Reduces lipid hydroperoxides to alcohols and reduces H$_2$O$_2$ to water</td>
</tr>
<tr>
<td>Carbonic anhydrase</td>
<td>Zn</td>
<td>Catalyst of H$_2$CO$_3$ equilibrium</td>
</tr>
<tr>
<td>Carboxypeptidase</td>
<td>Zn</td>
<td>Hydrolysis of peptide bonds at carboxyl terminal</td>
</tr>
<tr>
<td>Alcohol dehydrogenase</td>
<td>Zn</td>
<td>Dehydration of alcohol</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>Zn</td>
<td>Hydrolysis of phosphate esters</td>
</tr>
<tr>
<td>Metallothioneins</td>
<td>Zn, Cu, Cd</td>
<td>Metal storage and insertion into apoenzymes</td>
</tr>
<tr>
<td>Calcineurin</td>
<td>Zn, Fe</td>
<td>Phosphorylation</td>
</tr>
</tbody>
</table>
Most metals in these systems exist in the form of metal-protein and/or metal-sugar complexes with low concentrations being present in the form of unbound metallic ions (Lammers et al. 1994; Bandwar et al. 1996). However, there is the contention that the belief in unbound transition metal ions, such as zinc, that are available as “free” ions is erroneous (Maret 2000). Furthermore, at physiological pH, zinc ions (along with other transition metals) will precipitate as hydroxide complexes or bind to the myriad of organic ligands in tissue (Frederickson et al. 2005). In order to maintain consistency with the literature, histochemically reactive metals shall be referred to as unbound metal ions in this thesis.

Much of the information on elucidating the roles of unbound metal ions in the CNS was carried out using various histochemical methods such as “staining” (Sumi and Suzuki 2002). Morawski et al. (Morawski et al. 2005) have coined, “the gain in brain is mainly in the stain” – a humorous but key phrase characterizing neurodegenerative disease. For example, the anatomical evidence for the high concentrations of unbound ionic Zn in synaptic vesicles first arose from dithiozone staining, which delineates areas relatively high in concentrations of unbound Zn (Okamoto and Hashimoto 1944). Furthermore, experiments in hippocampal slices first demonstrated that Zn was synaptically released in a Ca-dependent fashion (Assaf and Chung 1984; Howell et al. 1984).

To be able to detect the bound metal ions, a substitution reaction with a chelating agent is required before they may be stained. However, metals with relatively high stability constants such as Cd-thionein (a metallothionein) are not well stained, therefore reducing the sensitivity of this type of assay for their detection. Generally, the conventional histochemical methods for staining metals were too insensitive and non-specific, especially for bound metals, which were deemed to be “invisible” to most cytological stains (Sumi and Suzuki 2002). Few histochemical staining reagents for these
types of metals have been reported and several technical difficulties with staining sensitivity and specificity have been pointed out for these techniques (Sumi and Suzuki 2002). For example, because of their similar chemical behavior, it is difficult to detect Cd in the presence of Zn by a staining reaction. Nolan et al. (Nolan and Lippard 2005) have identified the need for new Zn imaging tools with high sensitivity, selectivity and temporal fidelity in order to understand Zn neurobiology. ICP-SF-MS possesses these desirable features.

An important class of proteins involved in metalloneurochemistry are the MTs (Aschner et al. 1997). Metallothioneins (MTs) are a group of metal-containing (Cd, Cu and Zn) proteins, expressed in tissues and organs. The main functions of MTs are Cu and Zn homeostasis, detoxification of heavy metals and possibly metal transport, particularly in Cu-loaded animals (Evering et al. 1991). An immunocytochemical study of liver and kidney MT in Cu toxicosis in rats has confirmed the role of MT in Cu storage and adaptation to the metal. However, it is not clear whether brain MT plays a major role in metal detoxification, as does hepatic and renal MT (Evering et al. 1990). Although the precise physiological role of MT remains uncertain, it has been established that these proteins provide a protective effect within the mammalian CNS (Chung and West 2004), and are up-regulated in response to increased metal concentrations. For example, Cu, Hg, Ni and Ag are known inducers of MTs (Yamada and Koizumi 1993).

As the CNS is an extremely complex structure, with an estimated 30,000 mRNA species (Sutcliffe 1988), and one-third of the mammalian genome is dedicated exclusively to its function (Bantle and Hahn 1976), it is not unreasonable to believe that it may have developed unique processes for transporting, compartmentalizing, releasing, and utilizing trace metal elements.
1.1.4 Physical methods used to unravel trace metal elements in metalloneurochemistry


Atomic spectrometric techniques (such as AAS, ICP-AES and ICP-MS) are generally highly sensitive and so are used to measure the total elemental content within a sample, although they may be affected by the sample matrix (Brown and Milton 2005). X-ray (XRF and PIXES) and nuclear techniques (NAA) offer relatively low limits of detection (LODs) and are unaffected by sample matrices. As their principles of
operation are fundamentally different from those of the other analytical techniques, they are unlikely to be prone to the same systematic biases and so are valuable in method-comparability studies (Brown and Milton 2005). However, they are expensive, have a relatively low throughput and so can be difficult to calibrate (Brown and Milton 2005). Atomic spectrometric techniques (in particular ICP-MS) provide access to the lowest LODs currently available in routine analytical chemistry (Brown and Milton 2005). Moreover, they provide analysis of the total amount of element present in the sample regardless of the matrix or the environment of the target element (Baffi et al. 2002). Fundamentally, atomic spectrometric techniques operate in a similar fashion. A sample is atomized, ionized and its composition determined by the electromagnetic or mass spectrum generated. Overall, ICP-MS offers the “best” characteristics of all the atomic spectrometric techniques in terms of sensitivity, LOD, throughput and multi-element measurement (Brown and Milton 2005). ICP-MS also has a wide concentration and detection range and so quantitative results are obtained without having to recalibrate the instrument. A wide detection range also reduces sample-handling requirements and so minimizes potential errors as well as reducing analytical times by allowing samples with varying elemental concentrations to be analyzed simultaneously. For example, ICP-MS is capable of measuring concentration ranges from low ppt to high ppm.

The detection range and LODs of several analytical techniques for trace analysis in liquids are summarized in Figure 2 (Becker 2005). The following discussion focuses on the seminal investigations of trace metal analysis of the CNS using these methods.
Atomic spectrometric techniques, particularly AAS, have made the largest contribution to the quantification of trace metal elements in the CNS (Lowry et al. 1946; Eichelberger et al. 1949; Ames and Nesbett 1958; Colburn and Maas 1965; Hanig and Aprison 1965; Hanig and Aprison 1967; Donaldson et al. 1973; Rajan et al. 1976; Glynn et al. 1995; Gómez et al. 1997; Guo et al. 1999; Galván-Arzate et al. 2000). In earlier studies, Rajan et al. (Rajan et al. 1976) have determined the levels of Cu, Fe, Zn, Mg and Ca in the synaptosomal myelin and mitochondrial fractions of the rat brain by AAS. In vivo studies were also undertaken to investigate the effect of metal chelating agents on the sub-cellular distribution of the metal ions. More recently, Gómez et al. (Gómez et al. 1997) and Glynn et al. (Glynn et al. 1995) have performed trace metal analysis of rat brain tissue after the animals had been exposed to Al-dosed drinking water and Galván-Arzate et al. (Galván-Arzate et al. 2000) have investigated the effects of TI distribution in rat brain regions after sub-lethal doses of its compounds. Guo et al.
(Guo et al. 1999) have measured Fe and Zn in normal human brain tissue using AAS. The content of Fe in gray matter was greater than that in white matter, whereas the content of Zn in gray matter was comparable to that in the white matter with the Fe content overall found to increase with the increasing of age.

Thin slices of human brain tissue were analyzed by energy dispersive XRF with the distribution of elements being studied using micro-beam XRF in order to determine its suitability in studies on the role of trace elements in selected neurological diseases (Boruchowska et al. 2002a). Ide-Ektessabi et al. (Ide-Ektessabi et al. 2004) have employed XRF using synchrotron radiation to measure the distribution of trace metals (Ca, Fe, Zn and Pb) in brain tissues from diffuse neurofibrillary tangles with calcification (an atypical dementia) patients. Boruchowska et al. (Boruchowska et al. 2002b) have used XRF and PIXES for the detection of 14 elements in lyophilized human brain tissue (white and gray matter) in order to compare the two techniques for qualitative and quantitative analysis of biomedical materials. Ishihara et al. (Ishihara et al. 2003) have provided clear images of Ca, Fe and Zn within certain single neurons in the brain tissues affecting AD obtained using synchrotron radiation XRF. In addition, these results demonstrated a possibility of neuronal degeneration due to the imbalance of cellular metal elements in neurons of brain tissues affected by AD.

The PIXES technique was used for localized analysis of trace elements in the white and gray matter of human brain samples taken from autopsy patients aged from 17 to 88 years of age (Boruchowska et al. 2001). Elevation of Zn concentration with age of patients was observed for white matter and elements such as S, K, Ca, Mn, Fe, Cu and Zn exhibited higher mass fractions in gray matter than in white matter in all cases. Tapper et al. (Tapper et al. 1991) have determined a range of elements (K, Ca, Mn, Fe, Cu, Zn, Se, Br and Rb) in human malignant and normal brain tumors using macro- and
micro-PIXES analysis. Lierde et al. (Lierde et al. 1997) have examined specimen mass loss, major element loss, and alterations in minor and trace elements during nuclear microprobe bombardment of various types of human brain tissues. Also, Kemp et al. (Kemp and Danscher 1979) have used PIXES to analyze 13 elements including Zn, Rb, Fe, Pb and Ni with fixed and unfixed samples of the hippocampus, neocortex, amygdala and spinal cord of male Wistar rat brains. More recently, Morawski et al. (Morawski et al. 2005) have used PIXES to compare the neuronal Fe concentrations in human brain sections of healthy and Parkinsonian brain tissue.

Thirty elements in seven rat brain regions of female adult Wistar rats were determined by NAA (Chan et al. 1983). Specifically this group concentrated on the hypothalamus, striatum, mid-brain, cerebral cortex, pons and medulla, hippocampus and cerebellum. Andrási et al. (Andrási et al. 1995) have determined the average values of Fe and Zn in ten normal human brain regions, which showed a non-homogenous distribution of these elements. Falnoga et al. (Falnoga et al. 2000) have determined Hg, methyl-Hg, Se, Cd and Pb content in brain tissue from retired Idrija Hg mineworkers using a combination of NAA, Cold Vapour AAS and Electrothermal AAS in autopsy samples (hippocampus, cortex cerebellum, nucleus dentatus, endocrine glands and kidney cortex). Human brain samples were taken from the superior frontal gyrus, superior parietal gyrus and medial temporal gyrus of normal and AD subjects in order to determine Na, K, Fe, Zn, Se, Br, Rb, Ag, Cs, Ba and Eu using NAA (Panayi et al. 2001). Bromine showed the greatest difference, which was found to be significantly elevated in the cortex of AD brains compared to those of the normal brains (Panayi et al. 2001). Civit et al. (Civit et al. 2000) have measured 20 minor and trace elements in some intracranial tumors using NAA. Cutts et al. (Cutts et al. 2001) have performed elemental analysis of AD brain tissue samples taken from both left and right
hemispheres of three regions of the cerebrum (namely the frontal, parietal and occipital lobes for both Alzheimer and normal subjects) using a combination of PIXES and NAA. In addition, Positron Emission Tomography (PET) was utilized to detect regional cerebral metabolic rates of glucose.

Takahashi et al. (Takahashi et al. 2001) have used ICP-AES to measure P, K, Na, Mg, Ca, Fe, Zn, Cu, Rb, Al, Mn, Sr, Mo, Co, Pb, Cs and Ca using the whole brain of Sprague-Dawley rats. A combination of techniques (i.e. ICP-AES and NAA) were utilized to determine Na, K, Mg, Ca, P, S, Al, Cu, Mn, Sr and Zn in nine different regions from both hemispheres of normal human brains (Andrási et al. 1995). Rahil-Khazen et al. (Rahil-Khazen et al. 2002) have analyzed 14 elements in autopsy tissue samples from the brain front lobe, cerebellum, and other organs. In most tissues the concentrations of the essential trace elements followed the order Fe>Zn>Cu>Mn>Se>Cr>Co. The frontal lobe recorded the greatest Cu deposition while the cerebellum accumulated the most Cd in older subjects.

Recently, ICP-MS has found its way into trace metal analysis of biological materials because it is a multi-elemental technique (i.e. most all elements of the periodic table can be detected with one single analysis). In addition, the sample throughput is high (e.g. approximately four minutes scan time per sample with up to 70 elemental determinations) (Jakubowski et al. 1998). Moreover, it is potentially accurate and precise (linearity over a large range; that is, greater than six orders of magnitude). Lastly, the detection limits are very low (pg ml\textsuperscript{-1} range) (Marchante-Gayón et al. 1999). To many researchers ICP-MS is the clear successor to AAS. The major weakness of low-resolution ICP-MS is that it suffers from mass spectral interferences by polyatomic species; thus elements such as arsenic cannot be resolved without mathematical
corrections or by use of front-end reaction schemes (Moens 1997). However high-resolution ICP-SF-MS solves this drawback.

Currently there are available both low- and high-resolution instruments, with the difference between the two primarily lying in their mass spectrum filtering technology (i.e. quadrupole versus sector field). The most ubiquitous low-resolution instrument is the Inductively Coupled Plasma Quadrupole Mass Spectrometry (ICP-Q-MS), whereas the high-resolution instrument is denoted by ICP-SF-MS (the latter only being more recently deployed in metalloneurochemistry studies). An account of CNS studies using ICP-Q-MS follows.

Some of the trace metal determinations of the CNS using ICP-Q-MS include a comparative study with NAA of Al in the human brain (Andrási et al. 1996). Fujimura et al. (Fujimura et al. 1992) have measured Al, Sc, V, Cr, Mn, Cu, Rb, Sr and Mo in cerebellum, brain stem regions, hypothalamus, mid-brain combined with thalamus, striatum, hippocampus and cerebral cortex, using male Wistar rats exposed to one to three periods of restraint conditions, namely at 15, 30 and 60 minute stress periods. Moreover, Saito et al. (Saito et al. 1995) have measured Cu, Mg, Mo, Rb, and Zn in the same brain regions using Long-Evans Cinnamon and Long-Evans Agouti rats. Gélinas et al. (Gélinas et al. 1992) have determined the normal inorganic contents of brain and other organs from healthy Sprague-Dawley rats. Concentrations of Cd and Zn in different brain tissue regions of AD subjects and from subjects with senile involute cortical changes were determined using ICP-Q-MS (Panayi et al. 2002). Samples were taken from both brain hemispheres of the superior frontal gyrus, the superior parietal gyrus, the medial temporal gyrus, the hippocampus and the thalamus. Srivastava et al. (Srivastava and Jain 2002) have performed elemental analysis of human brain tissues in two regions of the AD brain, the parietal cortex and cerebellum, and compared them
with age-matched controls. Their analysis showed the differential distribution of some trace metals in the two regions of the brain. More recently, Jackson et al. (Jackson et al. 2006) have determined the distribution of Cu, Fe and Zn in whole rat brain sections (100 µm-thick) using laser ablation ICP-Q-MS (LA ICP-MS). Using a 60 µm laser spot size, a whole rat brain thin section of approximately 1 cm² was effectively mapped for the above metals. Bishop et al. (Bishop et al. 2007) monitored alterations in Fe metabolism following re-oxygenation of hypoxic mouse brain tissue using ICP-Q-MS. Hare et al. (Hare et al. 2010) constructed three dimensional maps of Fe, Zn, Cu, Mn and P in a 6-hydroxydopamine lesioned mouse brain using an LA ICP-MS imaging method (known as elemental bio-imaging). Serial consecutive sections were ablated from the same animal to produce the three dimensional maps. This elemental bio-imaging has been further utilized to construct a 46-plate reference atlas by aligning quantified images of metal distribution (Fe, Cu and Zn) with corresponding coronal sections from the Allen Mouse Brain Reference Atlas (Hare et al. 2012).

1.2 ICP-SF-MS methodology

The low detection limits of the ICP-SF-MS, together with clean preparation routines, allows the accurate determination of trace metal elements at levels as low as a few pg ml⁻¹ without sample pre-concentration (Marchante-Gayón et al. 1999). However, this feature becomes untenable if the prevention of contamination is compromised during sample collection, storage and preparation stages.

Contamination is a ubiquitous problem in trace element analysis since it potentially occurs at all stages from the collection of the original sample through to the final measurement. Contamination may be of two types namely, positive or negative.
The former refers to extraneous contamination such as the gain of actual analyte itself from the environment (e.g. laboratory ware, laboratory air, reagents *etc.*) or by the gain of a substance, which may cause interference such as the enhancement of the analyte in the determination process (e.g. may assist in the preferential ion generation *etc.*) For example, Rodushkin *et al.* (Rodushkin *et al.* 2000) have investigated contamination from the leaching of blood-storage glass tubes and found that elements such as Al, B, Ba, Ce, Co, La, Li, Nd, Pr, Si, Sm, Tb, Th, Ti, U and V increased the blood concentration more than five-fold. On the other hand, negative contamination refers to the loss of the analyte (e.g. adsorption onto the container walls). However, an initial negative contamination may result in subsequent measurements as a positive contamination. For example, well-known memory effects involve successive accumulation on instrumental surfaces, resulting in the degradation of detection limits (Moreton and Delves 1998).

In order to control contamination, only acid-washed polyethylene, polypropylene and Teflon laboratory ware are generally used. Glass, rubber and metal materials are avoided where possible, to prevent leaching and/or adsorption of trace metals (Diemer *et al.* 2001).

Airborne contamination from dust particles during sample treatment is significant since it is within the operational ng g\(^{-1}\) concentration range and so must be minimized (Diemer *et al.* 2001). Air cleaning and filtration is a fundamental requirement for a significant range of reliable trace element determinations and so most ICP-SF-MS investigations are conducted employing laminar flow hoods and/or laboratories, which are maintained at positive pressure, with HEPA filters installed. For example, Lamberty *et al.* (Lamberty *et al.* 1997) have described an ultra-clean chemical laboratory built at
the Institute for Reference Materials and Measurements designed to allow reliable, contamination-free sample preparation prior to inorganic elemental or isotopic analysis.

1.2.1 Sampling

In the past, most trace element analyses were performed on biological materials that were easy to obtain and that were considered traditional indicators of health conditions or disorders. Therefore, mass spectral analysis centered on urine, faeces, hair and blood or fractions of it (Moens 1997). There were significantly fewer investigations involving trace multi-elemental analysis of tissue.

A major difficulty when preparing tissues for analysis is the interference from such components as connective tissue, blood vessels, fat, residual blood and extracellular fluids. These components differ significantly in their trace element composition, thereby being a source of variation in analysis. Hence, care must be taken in order to avoid these sources of contamination during sub-sampling.

A variety of brain tissue sampling and sub-sampling techniques (including various combinations) have been used over the years for example: homogenization of whole brain tissue or excision of specific brain tissues regions; freeze-drying; fractionation of different tissue types; lyophilization; perfusion and fixation. The usage of the above techniques is dictated by issues such as initial sample amount, potential contamination, analyte loss and more importantly, the expression of the data within the context of the method employed.
1.2.2 Sample preparation

Prior to analysis using ICP-SF-MS, solid samples are required to undergo a form of digestion/dissolution (Subramanian 1996). Biological samples are complicated by a high protein/fat content, which may cause blockages of the nebulizer, torch tube and sample/skimmer cones. Additionally, high concentrations of organic solids change the properties of the argon plasma, which may also adversely affect the detection limits. This problem is particularly pronounced when the content of dissolved solids is greater than 0.2% (Subramanian 1996). In order to achieve accurate and reproducible results it is necessary to oxidize completely the organic matrix of biological samples prior to analysis by the ICP-SF-MS (Friel et al. 1990; Mingorance et al. 1993). This is achieved by oxidative conversion to CO₂ and H₂O.

In the majority of recent ICP-SF-MS studies, the most common approach for sample preparation involves “wet-ashing” techniques, followed by dissolution and dilution of the residue to a specific volume prior to analysis (Subramanian 1996). Microwave digestion is a widely used method for this purpose. It is found to be more reproducible, accurate, and less time consuming than conventional digestions utilizing hot plates (Kingston and Jassie 1988). The microwave systems maintain low blank levels, because only small volumes of reagents are required (i.e. typically one to five ml). Furthermore, they are also well suited for samples that are difficult to mineralize (Krachler et al. 1996). Mixtures of HNO₃ and H₂O₂ have often been successfully applied for MW-assisted tissue digestion (Engström et al. 2004). Nitric acid is preferred to other mineral acids since it produces the least amount of polyatomic interference (Subramanian 1996). For example, HCl and H₂SO₄ acids introduce polyatomic ions such as ArCl⁺ and SO⁺, respectively. Hydrogen peroxide has been found to be useful as an auxiliary reagent to HNO₃ as it increases the oxidizing power of the digestion, thus
minimizing the volume of concentrated HNO$_3$ required. Nitric acid concentrations higher than $ca\ 1.5\ \text{mol. l}^{-1}$ ($ca\ 7\%\ \text{v/v}$) cause rapid and severe corrosion of the sample and skimmer cones (Krachler et al. 1996).

1.3 ICP-SF-MS - a bio-analytical tool for metalloneurochemistry investigations

The determination of physiologically and toxicologically important trace and ultra-trace metals in animal tissue is difficult for ICP-Q-MS because in addition to interfering species such as oxides and argides, many species occur that originate from the matrix elements C, Na, P, S, Cl, K and Ca. High-resolution capability of ICP-SF-MS ($M/\Delta M = 10,000$) therefore offers a convenient way to eliminate most spectral interferences without further dedicated front-end or mathematical stripping procedures (Jakubowski et al. 1998). Hence even difficult elements in terms of spectral interference such as arsenic can easily be detected using ICP-SF-MS.

With rapidly increasing industrial development, trace metal analysis has received more attention. The main driving force in this development is the increasing demand to quantify at lower detection limits, down to previously inaccessible levels in both trace analysis and microanalysis (Stuewer and Jakubowski 1998). The ICP-SF-MS instrument is capable of performing rapid and simultaneous measurements on a wide range of elements (which can significantly increase the information available for kinetic models in human nutrition and toxicology) (Patriarca 1996). Additionally, this technique is highly sensitive and so it requires only small amounts of sample (e.g. typically low mg level). It is ideally suited for analyzing most trace metal elements in biological matrices. Nevertheless, as it has only been recently utilized for biomedical investigations methods need to be validated and benchmarked in order that the accuracy
of this technique is appropriately documented. It should also be noted that due to the high instrumental stability of ICP-SF-MS, isotope ratios can be measured with relatively high precision (Jakubowski et al. 1998). This allows for the use of stable isotopes as tracers in order to study trace metal metabolism, thereby foregoing the need for radioisotopes (Patriarca 1996). A full description of the strengths and weaknesses of ICP-SF-MS as well as suitable methods are given in Chapter Two.

To date only eight publications have investigated the CNS using ICP-SF-MS even though it is anticipated that because of its sensitivity, resolution and universality this biochemical tool will become more commonplace in the near future. Gellein et al. (Gellein et al. 2003) have determined Cd, Co, Cu, Fe, Mn, Rb, V and Zn in brain tissue of ALS and Parkinsonism-dementia complex patients. The concentrations of Cd were significantly elevated both in gray and white matter in ALS, but not in Parkinsonism-dementia complex patients. The concentrations of Zn were elevated for both patient groups, in the gray and white matter, although only the difference in gray matter for and Parkinsonism-dementia complex was significant. Prange et al. (Prange et al. 2001) developed a new approach for the speciation of MTs in human brain cytosols. The method was performed using CE coupled with ICP-SF-MS to detect Cu, Zn, Cd and S MT isoforms. Engström et al. (Engström et al. 2004) have determined up to 68 elements in rabbit brain tissue along with various other organs and species. Finally, Becker et al. (Becker et al. 2005c) have utilized LA ICP-SF-MS to measure Cu, Zn, P and S distribution in thin sections of the Caudatus putamen of a male F344 Fisher rat brain hemisphere for the detection of a small-sized tumor (thickness: 20 μm) and in an additional study (with the added elements Fe, Th and U) analyzed thin sections of human hippocampus (Becker et al. 2005d). The analysis yielded a heterogenous distribution for P, S, Cu and Zn in both studies. With respect to the latter study, Th and
U were more homogeneously distributed. Additional CNS studies conducted by these workers utilized LA on human brain proteins after isolation by 2-D gel electrophoresis (Becker et al. 2004; Becker et al. 2005a).

Table 1.3 compares some multi-elemental studies of whole rat brain tissue. Generally, the extent of the elemental values offered in the literature and the disparity of the methods employed do not allow results to be readily compared in rats.

The elemental concentrations recorded by the different investigators were of similar orders of magnitude in the case of As, Co, Cu, Fe, Mn, Se and Zn (Chan et al. 1983; Leblondel et al. 1986; Gélinas et al. 1992; Gómez et al. 1997) The highest variations amongst these workers were for Al, Co, Cr and V (Chan et al. 1983; Leblondel et al. 1986; Gélinas et al. 1992; Gómez et al. 1997).
Table 1.3 Comparison of elemental concentrations (wet weight µg g⁻¹) of whole rat brain tissue at 90% confidence limits.

<table>
<thead>
<tr>
<th>Element</th>
<th>Gelinas et al. ³</th>
<th>Chan et al. ²⁄⁴</th>
<th>Leblondel et al. ³</th>
<th>Gómez et al. ³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Al</td>
<td>0.568 ± 0.069</td>
<td>5.61 ± 1.11</td>
<td>NA</td>
<td>0.02 ± 0.04</td>
</tr>
<tr>
<td>As</td>
<td>0.092 ± 0.006</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Cd</td>
<td>0.001 ± 0.000</td>
<td>ND</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Co</td>
<td>0.002 ± 0.001</td>
<td>0.034 ± 0.009</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Cr</td>
<td>0.214 ± 0.014</td>
<td>ND</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Cu</td>
<td>2.33 ± 0.12</td>
<td>3.45 ± 0.65</td>
<td>1.94 ± 0.20</td>
<td>4.51 ± 1.31</td>
</tr>
<tr>
<td>Fe</td>
<td>35.4 ± 1.5</td>
<td>15.5 ± 4.1</td>
<td>11.4 ± 1.0</td>
<td>22.9 ± 1.8</td>
</tr>
<tr>
<td>Hg</td>
<td>ND</td>
<td>ND</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Mn</td>
<td>0.279 ± 0.016</td>
<td>0.191 ± 0.045</td>
<td>0.282 ± 0.021</td>
<td>0.51 ± 0.04</td>
</tr>
<tr>
<td>Se</td>
<td>0.104 ± 0.012</td>
<td>0.207 ± 0.038</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>V</td>
<td>0.025 ± 0.004</td>
<td>0.459 ± 0.113</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Zn</td>
<td>9.73 ± 0.40</td>
<td>7.37 ± 1.17</td>
<td>10.8 ± 0.7</td>
<td>16.7 ± 2.6</td>
</tr>
</tbody>
</table>

1) ND = Not Detected; NA = Not Analyzed.
2) The average concentration of 7 (Chan et al. 1983) brain regions, respectively.
3) Male, Sprague-Dawley. 4) Female, MRC Porton. 5) Male, Wistar.

1.4 Metal Speciation Incorporating ICP-SF-MS

The importance of metal speciation is now well recognized, given the steady increase in the number of publications produced on this subject over the past 25 years (Welz 1998). Speciation refers to the determination of the different chemical forms of a particular element. In many environmental, biological, geological and biomedical processes, the discovery of the form in which a given trace metal element exists is as important as the determination of its total content. This may be largely attributed to the
relationship established between the chemical form of an element, its toxicology and mobility. While the reporting of total trace metal element levels is driven for the most part by legislative and/or economic considerations, the information delivered is incomplete and in some instances misleading. For example, inorganic As is extremely toxic to human beings, whereas arsenobetaine (present in shellfish) appears to be relatively non-toxic. Chromium (III) is a necessary nutrient, while Cr (VI) is highly toxic (Guertin 2005). Thus, a distinction between different species of the same trace metal element is of paramount importance in determining such critical criterion as its bioavailability.

The investigation of trace metal speciation was motivated in part by environmental concerns after a high incidence of Hg poisoning in Minamata Bay, Japan in the 1950s (Donard and Caruso 1998). In fact much of the research conducted in this area to date has been environmentally based within the context of monitoring pollution that may ultimately result in human exposure (Apostoli 1999; Szpunar and Lobinski 1999). Major target species have included organometallic compounds (e.g. methylmercury butyltin), redox systems (e.g. Cr (III)/Cr (VI), As (III)/As (V) etc.), and complexes of metals and metalloids with biomolecules such as MTs (Apostoli 1999; Bettmer 2002). Metal speciation has provided information for defining and evaluating emission sources, the release mechanisms and the degree of interaction with environmental systems, all of which are species-dependent (Olesik et al. 1998; Apostoli 1999).

Speciation resolution involving ICP-SF-MS methods has been found to be very useful in view of its sensitivity and suitability for on-line detection after chromatographic separation (Vela et al. 1993). For example, Willie et al. (Willie et al. 1997) have determined inorganic- and total-Hg content in various marine CRMs (i.e.
dogfish muscle and liver, and lobster hepatopancreas) using a chemical reaction to volatilize methyl-Hg from the tissue, thereby leaving inorganic Hg to be quantified by ICP-Q-MS.

It should be pointed out that the main intrinsic disadvantage of unadulterated atomic spectrometry is that it involves the decomposition of a sample into its constituent atoms, thereby destroying any speciation information. Therefore, speciation can be determined by combining atomic spectrometry with species-specific front-end techniques or by modifying the instrument substantially. Hieftje (Hieftje 1998) has outlined three approaches to speciation, which are as follows:

1. The coupling of an atomic spectrometric method with an auxiliary instrument that allows speciation (e.g. chromatography);

2. The use of a chemical or physical pre-treatment before atomic spectrometry is performed (e.g. precipitation, volatilization, and extraction);

3. The use of a spectrometric source, which may be operated alternately in two modes, one of which produces free atoms and the other of which generates molecules or molecular fragments in which the analyte elements initially existed (e.g. the use of ESI with a range of collision energies and desolvation conditions).

The most often applied chromatographic technique is High Pressure Liquid Chromatography (HPLC) (Heitkemper et al. 1989; Subramanian 1996) using various separation principles (e.g. reverse phase, ion exchange etc.) Jakubowski et al. (Jakubowski et al. 2001) have developed an HPLC procedure for the speciation of organic Se compounds in herring gull eggs followed by multi-elemental analysis using ICP-SF-MS. Zheng et al. (Zheng and Hintelmann 2004) have described a hyphenation
technique between HPLC and ICP-SF-MS for ultra-trace arsenic speciation analysis. The method was applied to the detection of anionic and cationic arsenic compounds in freshwater fish samples from Moira Lake in Canada, which has high levels of arsenic. Tetramethylarsonium ion was detected in freshwater fish for the first time. Truscott et al. (Truscott et al. 2001) have determined different oxidation states of actinides (Th, U, Am, Np and Pu) in human lung (NIST 4251) and Rocky Flats soil (NIST 4353) CRMs using chelation ion HPLC coupled to ICP-SF-MS. A method for the quantitative analysis of Fe speciation (heme and non-heme fractions) in raw and cooked beef steak by HPLC coupled to ICP-SF-MS was reported (Harrington et al. 2001). Nagaoka et al. (Nagaoka et al. 2004) have examined the binding patterns of V to transferrin in healthy human serum using HPLC/ICP-SF-MS. A method was developed by Bouyssiere et al. (Bouyssiere et al. 2004) that allowed the Ni species in cytosols of normal and malignant human colonic tissues to be compared. It was based on the hyphenation of sequentially-applied anion-exchange and size-exclusion chromatography using ICP-SF-MS as the detector.

Marchante-Gayón et al. (Marchante-Gayón et al. 1999) have pointed out the eventual requirement for confirmation techniques and methods in order to be certain of the identity of the species. Identification of a new species, chromatographically separated in real samples (and using ICP-SF-MS as the detector), relies only on the observed retention time. This parameter would not provide sufficient identification because it depends on the chromatographic conditions. Furthermore, an adequate standard (appearing at the same retention time in the same conditions) would need to be available for final identification of a given species in the samples. A suitable confirmation technique would therefore be carried out using a molecular technique, such as ESI-MS. For example, Nischwitz et al. (Nischwitz et al. 2003) have described
the extraction and characterization of trace metal species from porcine liver samples using online HPLC/ICP-Q-MS and offline HPLC/ESI-MS. Whilst ionization sources such as ESI and MALDI can be used for the identification of and determination of the nature of the bonding of metalloproteins, these techniques cannot provide direct quantitative analysis of them, therefore the combination of elemental and molecular techniques would necessarily complement each other.

Of the few studies related to metalloneurochemistry that have used ICP-MS for speciation, only four have employed the high-resolution ICP-SF-MS. The first study (Prange et al. 2001) describes the speciation of MTs in human brain cytosols using CE coupled with ICP-SF-MS. Isoforms of MTs were separated and the elements Cu, Zn, Cd and S were detected. The analytical procedure developed was used for the first time in comparative studies of the distributions of MT isoforms in brain samples taken from patients with AD and from a control group. Gellein et al. (Gellein et al. 2007) developed a method to study the protein binding patterns of trace elements in human cerebrospinal fluid. Protein fractions were separated using size exclusion HPLC and analysed for Cd, Mn, Fe, Pb, Cu and Zn off-line using ICP-SF-MS. The remaining studies (Becker et al. 2004; Becker et al. 2005a) employed a new strategy for the speciation of metalloproteins, which involved the direct laser ablation of protein spots off 2-D gels. Neilsen et al. (Neilsen et al. 1998) originally used this technique for the identification of Co binding serum proteins. Becker et al. (Becker et al. 2004) determined P, S, Si, Al, Cu and Zn in human brain proteins from AD patients. Phosphorous, Si and the metals were expressed as relative ion intensities with respect to S in protein spots. In a subsequent study, Becker et al. (Becker et al. 2005a) identified Cu-, Zn- and Fe-containing human proteins from Alzheimer brain samples. Isotopic enriched tracers (\(^{54}\)Fe, \(^{65}\)Cu and \(^{67}\)Zn) were doped to two-dimensional gels of separated
AD brain proteins after 2-D gel electrophoresis in order to study the formation of proteins containing Cu, Fe and Zn. Laser ablation ICP-SF-MS is most useful as a fast-screening technique for metal-containing proteins, which couples well with the high-resolution capability afforded by 2-D gel electrophoresis. However, a major disadvantage is the quantification of analytical data due to the shot-to-shot variation of the laser ionisation source and the absence of a well-defined standard (Becker et al. 2004).

The remaining studies involved the use of ICP-Q-MS. Richarz et al. (Richarz and Brätter 2002) have performed speciation analysis of protein-bound elements (Cu, Zn, Cd, Pb and Ag) in the cytosol of human brain using size exclusion HPLC/ICP-Q-MS. Post-mortem samples from AD brains and from brains of a control group were investigated to reveal changes in the trace metal distribution during the pathological process with an emphasis on MTs. Połeć-Pawlak et al. (Polec-Pawlak et al. 2004) have investigated the speciation of Al in neuroblastoma cells exposed to Al lactate by size exclusion HPLC/ICP-Q-MS and CE ICP-Q-MS.

1.5 Current Metalloneurochemical Investigations in Neuroplasticity

The main function of the CNS is the storage and transmission of information. This process is known as neurotransmission, the nature of which involves the propagation of electrochemical impulses through synaptic junctions in response to a chemical or mechanical stimulus. In some cases processing of these stimuli (depending on their consistency and strength) have the effect of motivating the brain to reorganize its neural pathways based on a new experience (e.g. learning, trauma etc.) This ability of the brain
to adapt an “experience” is known as plasticity or neuroplasticity and is the underlying concept of the study of the CNS’s biochemistry and physiology.

Approximately two decades ago, the adult brain’s neural network was considered as structurally fixed. Subsequent pioneering studies, which led to a significant shift in paradigm in the field of neuroscience, have since shown that this is not the case (Merzenich et al. 1983; Merzenich et al. 1984; Calford and Tweedale 1990; Pons et al. 1991). By studying simple models of brain injury (or in a general sense, experience-dependent plasticity), using the spinal- and brain-representations of the tactile/visual/auditory system of animals as models, plasticity can be “measured” thus leading to a greater understanding of the CNS and its adaptability and physiological time-course. Accordingly, neuroplasticity occurs under two primary conditions:

1. During “normal” brain development when the immature brain begins to process sensory information through to adulthood (i.e. developmental plasticity and plasticity of learning and memory);

2. As an adaptive mechanism to compensate for lost function and/or maximize remaining functions in the event of brain injury.

An example of the approach of a typical study of plasticity is the identification of anatomical pathways, namely, the formation of new synaptic connections, which represent the occurrence of events (Martin and Morris 2002). For example, Calford et al. (Calford et al. 2003) have showed that the placement of monocular lesions in the adult cat retina produced a lesion projection zone in primary visual cortex in which the majority of neurons have a normally located receptive field for stimulation of the intact eye and an ectopically located receptive field (displaced to intact retina at the edge of the lesion) for stimulation of the lesioned eye.
Although neuroplasticity has been widely studied since its initial discovery, the description of the underlying molecular mechanisms has lagged behind (Berardi et al. 2003). Some studies have emerged which involve Zn level flux. For example, Czupryn and Skangiel-Kramska (Czupryn and Skangiel-Kramska 2001b) have investigated the distribution of synaptic Zn after short-term (up to 48 hours) tactile deprivation of vibrissae in the barrel cortex of mice using histochemical staining. Land et al. (Land and Shamalla-Hannah 2002) have used histochemical localization of synaptic Zn to investigate normal postnatal development and experience-dependent plasticity of Zn-containing circuits in somatosensory barrel cortex of rats. Karakoc et al. (Karakoc et al. 2003) have investigated the effects of acute and chronic immobilization stress on the Zn, Cu and Fe levels of the temporal lobe, brain stem, spleen and liver tissues in rats using flame AAS. The animals in the acute stress group were put in cages once only for 120 minutes. With respect to the chronic stress groups the rats were kept in the cages daily for two and four hours, respectively, for five consecutive days. Their results showed that acute immobilization stress causes endogenous Zn and Cu release from the brain tissues. In the two hour chronic stress group, Fe levels increased significantly in the temporal lobe and brain stem and to a lesser extent also increase in the four hour chronic stress group.

Hurd et al. (Hurd et al. 1987) have measured the effects of three anti-convulsants (phenytoin, valproic acid, and γ-vinyl GABA) on trace elements of rat tissue and brain cortex using PIXES. The analysis revealed a decrease by all three drugs in Cu levels in brain cortex. A significant decrease in Se levels in brain cortex was measured with phenytoin and valproic acid. Moreover, the results were discussed in relation to the implication of imbalance in tissue metal levels in epilepsy.
Equally, some studies of neuroplasticity utilizing ICP-Q-MS have appeared. Gélinas et al. (Gélinas and Schmit 1994) have determined the inorganic contents of brain and other tissues from male spontaneously hypertensive rats and compared them with the same tissues from healthy male Sprague-Dawley rats. Percentage increases of the following elements in the brains of hypertensive rats were found: Na 18.8; As 22.8; Cr 15.4; Rb 99.4 and Zn 24.7%. The increase in As was attributed to an incomplete perfusion of the hypertensive tissues, since the erythrocytes (the main cumulative tissue for As) showed no significant variations. No explanation was found for the higher concentration of Cr. Increases of Rb in hypertensive tissues are correlated with those of K, but they are less pronounced. Only the brain tissues behaved differently with a 99.4% rise (while K is only 7.3% higher). Since Rb levels are usually uniform within members of a given species and are somewhat independent of exposure, the origin of this difference could be genetic. No relation could be found between the increase of 24.7% in brains and hypertension.

Fujimura et al. (Fujimura et al. 1992) have determined the concentrations of ten elements in seven brain regions of the restraint stressed rat with ICP-Q-MS. Rats were exposed to one of three restraint conditions (15, 30 and 60 min stress periods). A significant increase in Rb concentration and significant decreases in Mn and Cu concentrations were observed in some regions of stressed rats compared to those in control rats. On the relation between trace element concentrations and durations of the stress, rats exposed to long stress periods tended to have many significant changes of metal levels compared to those exposed to the short stressed period. The strong effects of stress were observed in the regions of the hippocampus, the midbrain and the thalamus.
Copper, Mn, Mo, Rb and Zn concentrations of seven brain regions in male Long Evans Cinnamon rats were determined using ICP-Q-MS before (four and ten weeks old) and after (twenty weeks old) the onset of jaundice (Saito et al. 1995). Long Evans Cinnamon rats have an abnormal Cu metabolism including excessive Cu accumulation in the liver, a reduction in the biliary excretion of Cu and a low level of serum Cu. These clinical features of Long Evans Cinnamon rats are similar to those of Wilson’s disease, a genetic disorder of the Cu metabolism in man (the excessive Cu accumulation in the brain is thought to cause neurological disturbances). Copper in the Long Evans Cinnamon rat brain was less concentrated in all regions at four weeks of age and in synaptosomal fractions at ten weeks, but conversely more concentrated in three regions at 20 weeks than in control rats. Furthermore, Mo and Rb in six regions at ten weeks of age and Mn at 20 weeks were more concentrated in the Long Evans Cinnamon rat brain than in control rats. These results showed that abnormal distributions of trace metals exist in the Long Evans Cinnamon rat brain before the onset of jaundice.

1.6 Scope of this work

The underlying theme of this thesis is the marriage of the two domains of analytical chemistry (instrumentation) with neuroscience. The purpose of this synthesis is to integrate quantitative physical measurement within analytical chemistry to supplement and expand on the traditional biochemical techniques, with respect to trace metal analysis of brain tissue.

This will be achieved by way of the processes enumerated below:

1. To develop and validate generic ICP-SF-MS methods for the sampling, sample preparation and multi-elemental analysis of brain tissue of rats in
order to benchmark/establish their normal or healthy trace metal concentration levels and distribution;

2. To investigate trace metal fluctuation (in particular Zn, Cu, Mn, Fe and Mg) in rat brain tissue after reduced sensory input by vibrissae removal using ICP-SF-MS;

3. To develop methods of screening for metalloproteins using a combination of LC-MS instrumentation (i.e. HPLC/ICP-SF-MS and HPLC/ESI-TOF-MS) and subsequently investigate protein-bound and unbound metal species in rat brain tissue (barrel cortex) after reduced sensory input by vibrissae removal.

These processes will take advantage of the highly quantitative features of ICP-SF-MS and its high sensitivity towards trace metal analysis. Not only will these characteristics assist in establishing the healthy or normal concentration ranges of trace metals in brain tissue and their roles in neuroplasticity, they will also prove invaluable as a rapid means to identify and quantify metalloproteins using their trace metal “signature/label”.

Chapter Two provides a description of an ICP-SF-MS and HPLC and their operation, with respect to this work. It will also describe the metalloprotein analysis methodology employed. The first process of this work is described in Chapter Three and will demonstrate that trace metals are unevenly distributed in the brain by measuring the elemental composition in eight different brain regions of healthy rats. Chapters Four and Five, which are concerned with the second and third process described above, will demonstrate that trace metals fluctuate in specific regions during
neuronal plasticity response and that these trace metals are protein-bound. Lastly, Chapter Six concludes this work and outlines future directions for investigation.
Chapter Two: Instrumentation and Metalloprotein Methodology

2.1 Introduction

Current neuroscience practise for the analysis of elements usually involves the use of staining techniques rather than instrumentation. While this allows the investigator to associate the analyte with biological function it is limited quantitatively, as pointed out in chapter one. On the other hand, the reverse is true with respect to the use of instrumentation. This limitation is somewhat overcome by the utilization of ICP-SF-MS in the current work, given its capability of accurately measuring elements in low mg level amounts of tissue.

In the current scope of work, it was necessary to measure several elements simultaneously under the same analytical conditions without any artifactual results in order to make meaningful comparisons. This necessity required an instrument with high selectivity and sensitivity. Consequently, this dictated that the instrument of choice be ICP-SF-MS.

Here an introduction to ICP-SF-MS theory and methodology is provided along with the validation of its performance. In addition, HPLC instrumentation and operation are also described along with the metalloprotein analysis methodology used in this thesis.

The techniques of ICP-MS, ICP-AES and AAS, currently available for measurement of elements in biological materials, share the same generic principles of operation. Liquid samples are aspirated into the instrument via a nebulizer, atoms are then de-solvated, volatilized, and ionized. After this point, the spectroscopic techniques AAS and ICP-AES differ significantly from ICP-MS in that energetic
wavelength emissions in the UV-visible spectrum from the ionization process are measured in the former and ionized isotopes are measured in the latter.

The atomic measurement technique ICP-MS has several advantages over its predecessors ICP-AES and AAS. While AES and MS techniques shared the same ionization process (i.e. ICP), the physical measurement of isotopes compared to wavelength emissions results in a less complicated spectral output as there are considerably fewer isotopes for a given element than there are emission lines. This reduction in inter-element interferences and therefore background contribution produces relatively lower detection limits. The advantage of the ICP technique over the AAS technique is the use of the atmospheric pressure argon (Ar) based plasma for increased sample atomization and ionization along with a dynamic range of 3-4 orders of magnitude. The acetylene based flame of an AAS can only reach temperatures up to approximately 2700°C compared to the inductive resonance based ICP source of 9700°C.

The key to the marriage of ICP with mass spectrometry is the interface region between the plasma and the high vacuum that allows the sampling and transport of ions representative of the solution being analyzed. The evolution (and increasing number of commercial manufacturers) of ICP-MS instrumentation, brought various modifications and improvements to the “front-end” technology of the instrument (such as collision cells and hydride generation) in order to minimize the inherent matrix- and argon-based interferences to the analyte of interest. With the advent of high-resolution sector-field mass spectrometry, these front-end modifications are unnecessary, as the resolution is available to overcome its predecessor’s (low-resolution ICP-Q-MS) limitations.
2.2 Inductively Coupled Plasma process

2.2.1 Inductively Coupled Plasma torch

The components necessary to create an ICP include a plasma torch, an radio frequency (RF) coil and a power supply. Figure 2.1 gives a detailed view of the plasma torch and RF coil relative to the mass spectrometer interface.

Figure 2.1 Cross-section of a plasma torch and RF coil relative to the mass spectrometer interface.

The plasma torch consists of three concentric tubes, which are made of materials that are transparent to the RF radiation (i.e. quartz). In Figure 2.1 these are shown as the outer tube, middle tube and sample injector.

As the temperature of the ICP is high (up to 10,000 K) the plasma is normally thermally isolated from the quartz walls of the torch by an outer (or cooling) flow of Ar which prevents the torch from melting. This cool gas is introduced tangentially and spirals in a vortex between the outer and middle tubes at a flow rate of ca 12-18 L min\(^{-1}\). This vortex flow both stabilizes and thermally isolates the plasma. The formation of the plasma itself is provided by a gas flow through the middle tube at approximately 1 L min\(^{-1}\). The sample injector transports the aerosol generated from the sample introduction
system using a flow of Ar gas also approximately 1 L min$^{-1}$. This gas flow is known as the nebulizer gas.

2.2.2 Inductively Coupled Plasma process

An ICP is formed when energy is transferred to a gas by means of an induction coil (or RF coil). For the plasma to be sustained by induction, a sufficiently large proportion of atoms must be ionized to make the gas conducting. The plasma forms above the sample injector and within the outer tube with its location being largely determined by the position of the induction coil.

The induction coil is part of a RF oscillator circuit, constructed from copper tubing and typically used at a frequency of 27 MHz (Browner et al. 1998). When an RF current flows in an induction coil it generates a rapidly varying magnetic field within the coil. This interaction (or inductive coupling) of the oscillating magnetic field with flowing gas generates the ICP.

Fundamental to the formation of an ICP, is that the motions of charged particles can be altered by a magnetic field. When a magnetic field is oscillating rapidly, the charged particles induce eddy currents within it. The fluctuating magnetic field couples with the plasma, which contains charged particles. These charged particles are accelerated, and energy is transferred from the particles to the gas atoms by collision. Since the plasma is at atmospheric pressure and the mean free path of the particles is small (ca 1 µm) (Taylor 2001), the rate of collision is high, causing a high degree of excitation (heating) and ionization. Highly ionized high-temperature plasma is then formed inside the inner tube of the tube, which is surrounded by the induction coil.
Since a gas is a poor conductor until it is ionized, the plasma must be initiated by being “seeded” with a sufficient supply of energetic electrons to ionize the argon. This can be achieved by the generation of a spark in the Ar stream using a Tesla coil. Electrons and Ar ions are then formed which absorb energy from the alternating field. Collisions between the accelerating Ar ions and electrons continue in a chain reaction to generate a deluge of charged particles to absorb the energy from the RF field. Once the electrons reach the ionization potential of the Ar, further ionization takes place, and a stable self-sustaining plasma is formed (Thompson and Walsh 1989) (the process of which is known as *inductive coupling*).

### 2.3 Thermo Fisher Element 2 ICP-SF-MS instrumentation

The Element 2 ICP-MS is an ICP double-focusing sector type mass spectrometer consisting of two mass analyzers in the form of an electromagnet and an electric sector analyzer (ESA). The Element 2 employs the reverse *Nier-Johnson* geometry (i.e. the electromagnet is positioned before the ESA), which is shown schematically in Figure 2.2 (ThermoElectron 1999).
2.3.1 Sample introduction

The function of the sample introduction system is to introduce a liquid sample to the instrument in the form of a fine aerosol. The higher surface area to volume ratio thus allows for a more efficient ionization in the plasma. Presented in Figure 2.3 is the standard inlet system for the Element 2 (also equipped with a CETAC ASX-500 autosampler) used throughout the work presented in this thesis.

Figure 2.2 Schematic of the Thermo Fisher Element 2 (ThermoElectron 1999).
The inlet system consists of the peristaltic pump, nebulizer, spray chamber, torch with sample injector, and the load coil and match box (the match box being a part of the RF generator).

The sample is normally pumped at a flow rate between \( \text{ca} \ 100 \ \mu \text{l min}^{-1} \) to 1 ml min\(^{-1}\) via a peristaltic pump which maintains a constant flow into the nebulizer. The nebulizer breaks up the liquid stream of sample into a fine aerosol via co-introduction with an Ar gas flow into the spray chamber. The spray chamber then removes any large droplets remaining, thereby ensuring that only small droplets enter the plasma. Throughout this work a double-pass Scott-type spray chamber was employed, in which the aerosol emerges from the nebulizer and is directed into a central tube running the whole length of the chamber. The droplets travel the length of this tube, where the large droplets (\( > 10 \ \mu \text{m in diameter} \)) fall out by gravity and exit through the drain tube at the
end of the spray chamber. The fine droplets (ca 5-10 μm in diameter) then pass between the outer wall and the central tube, where they eventually emerge from the spray chamber and are transported into the sample injector of the plasma torch.

2.3.2 Vacuum system

A basic requirement for mass spectrometry is the unimpeded movement of ions through to the detector, hence the need for an ultra high vacuum (UHV) system. In ICP-MS, ion generation occurs outside of the vacuum system in the ICP of the torch. The focusing, mass separation and detection must all take place in the UHV region in order to ensure that the mean-free-path length is longer than the physical size of the chamber. The UHV system, in the Element 2, reduces the pressure from atmospheric (in the plasma region) to a UHV of less than 10^{-7} mbar in the detector region.

2.3.3 Ion generation

The sample aerosol generated from the sample introduction system is transported through the sample injector in a stream of argon gas into the plasma. The sample aerosol is desolvated and the atoms are converted from solid particles to a gaseous state and then into a ground-state. Finally, the ground-state atoms are ionized by collisions with energetic electrons and Ar ions. As the ionization energy of the atmospheric pressure Ar ICP is 15.76 eV and the majority of elements have a first ionization energy considerably less than this value, the plasma will efficiently produce singly charged ions for most of the periodic table of elements. The ions generated are then directed to the interface region.
2.3.4 Ion extraction

The ion focusing system transports positive ions only from the interface region to the mass analyzer. Figure 2.4 shows the ion focusing system of the Element 2 (ThermoElectron 1999).

The interface region consists of two nickel cones and the gate (or skimmer) valve (which, when closed, seals the mass spectrometer from the outside environment). The ions initially pass through the sample cone, to the skimmer cone. The ions then emerge from the skimmer into the lens system.

An extraction lens then (maintained at -2000 V) extracts the positive ions out of the main gas stream in the cone region to the following lens stack by creating a charge gradient. Given that the ion beam is positive and the individual ions will repel one another, ion focusing is required to force the ions back into a focused beam and directed to the mass analyzer.

![Figure 2.4 Schematic of the Element 2 interface and ion focusing regions (ThermoElectron 1999).](image-url)
2.3.5 Mass analyzer

Using the reverse Nier-Johnson geometry approach to mass separation (Jakubowski et al. 1998; Stuewer and Jakubowski 1998), the ions are accelerated to 8 keV before they enter the mass analyzer. The mass analyzer is composed of an electromagnet and an electric sector analyzer (ESA). By combining a magnetic-sector mass analyzer with an ESA, a significant improvement in resolution is realized (ca up to 10,000 m/Δm for the Element 2). The mass resolution is controlled by the entrance and exit slits, both of which have three openings of three fixed widths. These widths give the three resolution settings for the Element 2: low-resolution, medium-resolution and high-resolution modes. The ICP-SF-MS can scan either by varying the magnetic field as a function of time or by holding the magnetic field constant and varying the voltage applied to the electrostatic analyzer.

A magnetic field serves to focus ions with diverging angles of motion before reaching the ESA (Figure 2.2). The magnetic field separates ions according to their ion kinetic energy and mass (or rather their momentum-to-charge ratio) by varying its field strength. After the ion focusing system has accelerated ions of mass \( m \) with a potential difference \( U_a \) into the magnetic sector field \( B \), all ions will travel through it with a radius of curvature, \( r_m \). The \( m/z \) may then be calculated using the following equation:

\[
m/z = B^2 r_m^2 / 2U_a \tag{2.1} \]

(Jakubowski et al. 1998).
As ions of identical mass don’t necessarily have the same energy, the ESA serves as a second stage analyzer separating ions by their kinetic energy and directing them onto the detector.

When the ion beam emerges from the intermediate slit it passes into the electric sector, where the electric potential maintained across it causes the ions to follow an arc and to separate according to their translational energies. Ions of the same energy are then brought to a line focus by using a focus and rotation quadrupole (which correct the focus and the beam rotation) to align the beam to the exit slit. Therefore, the ESA disperses ions with different translational energy whilst focusing ions of like energies (Johnstone and Rose 1996).

If $V$ is the potential through which the ions are initially accelerated and $E$ is the field in the electric sector, then equation (2.2) is obtained:

$$ R = \frac{2V}{E} \quad (2.2) $$

where $R$ is the radius of curvature of the ion path (Johnstone and Rose 1996). The two equations governing the motion of the ions through the electric sector are those relating their kinetic energy, $ZeV = \frac{mv^2}{2}$, and centrifugal force, $ZeE = \frac{mv^2}{R}$, where $v$ is the velocity of an ion of mass $m$ and number of charges $Z$, and $e$ is the charge on an electron. The combination of these two equations yields equation (2.2). Ions accelerated through a potential $V$ and passing through a uniform field $E$ have the same radius of curvature $R$ irrespective of $m/z$. Therefore, if the field $E$ is kept constant, the ESA separates ions according to their translational energies.
2.3.6 Detection system

The Element 2 uses a SEM. After the ions emerge from the ESA (through the exit slit) they pass through the deflection plates. The deflection plates are used to prevent the detector from being damaged by large mass peaks by diverting the ion beam. After passing through the deflection plates, the ions strike the conversion dynode held at -8 kV. From the impact of the ions, the dynode secondary electrons are generated and released from the surface of the dynode. The electrons are then attracted into the SEM where more electrons are liberated. The electrons are accelerated from one dynode to the next generating a pulse of electrons that are captured by the multiplier collector. The SEM itself has 19 dynodes giving a typical overall gain in the range of $10^6$.

2.4 Operation of Thermo Fisher Element 2 ICP-SF-MS

The Element 2 software controls and monitors all instrument functions for analysis by ICP-MS. This includes data acquisition, tuning and calibration. The software is divided into separate modules, with each module having a different application.

The following steps can outline the analysis sequence:

1. **Start-up** that involves ignition of the plasma and, tuning and calibrating the instrument (carried out by modules “Instrument”, “Tune”, and “Mass Calibration”).

2. **Creating a measuring program** where mass ranges, sampling times, resolution settings and measurement modes are specified (module “Method Editor”).
3. **Creating standard and internal standard files** stating the elements to be evaluated, their concentrations in the standard solutions, as well as the internal standard elements and their concentrations (module “Standard Editor”).

4. **Creating a sample sequence** *i.e.* order in which blanks, samples, and standards are to be analyzed (module “Sequence Editor”). “Sequence Editor” also associates tune, calibration, method and standard editor files with the samples to be analyzed.

5. **Starting measurements** (module “Sequence Editor”)

6. **Evaluating and displaying measurement data** (modules “Show”, “Result & Display”).

### 2.4.1 Tuning

After ignition of the plasma, the next step is to tune the instrument. The “Tune” module allows the operator to alter vital parameters of the instrument in order to optimize performance. The tuning procedure must be carried out before every analysis to ensure that the system is operating correctly. The tuning of the instrument is conducted separately for each resolution (although the altered parameters are saved in the same file).

The window of the “Tune” module gives a graphical representation of the major components of the instrument from the torch positioning, gas flows and plasma power (RF) settings, as well as the lens system (Figure 2.5). The six display panes allow for the “on-line” monitoring of the peaks. Additionally, mass segments may be plotted as a chromatogram trace for monitoring signal stability (e.g. Pane E and F).
Figure 2.5 Tune window of Element 2.

To tune the system, a suitable sample solution must be introduced. This means that, when tuning, mass peaks must be present in those ranges, which will be used in the subsequent analyses. Generally, a multi-element solution consisting of: Li, B, Na, Sc, Co, Fe, Zn, Y, Rh, In, Ba, Lu, Tl, and U (with each element present at a concentration of 1 ng ml\(^{-1}\)) is used.

The instrument is then tuned in low resolution mode for optimum sensitivity and stability across the mass range. The parameters concerned are the sample and auxiliary gas flows, torch position, plasma power and the lens parameters (ion focusing system). The elements Li, In and U are typically monitored for general multi-element determinations as they represent the start, middle and end of the mass range, respectively. For the 1 ng ml\(^{-1}\) solution, for example, a typical intensity for Li, In and U are \(10^5\), \(10^6\) and \(10^6\) counts per second, respectively with percentage relative standard deviations of < 1%. Table 2.1 shows the average instrument detection limits of the
Element 2 recorded over the duration of this work. These were monitored routinely in order to maintain the clean preparation routines already discussed in section 1.2.

Further tuning is then carried out in medium resolution and high resolution modes to optimize peak shape and resolution. The high-resolution lenses (rotation and focus quadrupoles) are used to accomplish this. For routine high-resolution tuning, $^{56}$Fe (MRM) and $^{39}$K (HRM) are monitored to ensure a baseline separation from their respective interferences of ArO and ArH. Additional parameters, such as SEM deflection and voltages, are altered upon installation of the instrument and then only on occasion (when the SEM begins to degrade). Once tuning is complete the new parameter settings are saved and the instrument is now ready to be calibrated.

2.4.2 Calibration

Mass calibration is the calibration of the magnetic field strength and the $m/z$ of the ions passing the field magnet against the reference voltage of the DAC used to set the field strength (i.e. mass calibration is required to calculate the DAC data from mass numbers and vice versa).

A multi-element solution, as described above, is used to carry out a mass calibration. By using this solution, the instrument can be calibrated over nearly the entire mass range (2-260 Daltons).
Figure 2.6 Element 2 Mass Calibration window.

The upper part of the Mass calibration window displays the MDAC spectrum (Figure 2.6). The spectrum to be displayed here must first be acquired, either with the “Instrument” or “Sequence Editor” modules. A calibration curve is also displayed in the window to check the quality of the mass calibration. The curve must be smooth and rise steadily up from a minimum on the left to a maximum on the right (translation table). A diagnostic display also reflects the quality of the mass calibration. The conversion error is calculated as follows: a MDAC value is calculated from a mass \( m_0 \). This DAC value \( i \) is converted back to a mass \( m_1 \). The relative difference \( (m_1 - m_0)/m_0 \) are plotted against the \( m_0 \). Any abnormal calibration point will create a peak in this curve, so incorrect mass entries are easily recognized. Once the calibration is complete, the system is ready for analysis.
2.4.3 Analysis

When conducting an analysis, the first step is to fashion a suitable measuring program which will determine the way in which a set of data will be acquired and evaluated. The parameters may be divided into three classes: (i) parameters relating to the elements to be measured: mass range (mass window) and isotopes (ii) parameters relating to data sampling: sample (dwell) time, samples per peak, replicates (runs × passes) (iii) parameters relating to the measuring method: resolution, scan mode (electric or magnetic), detection mode (analogue or counting), integration type, regression type.

The analyses (using a Thermo Electron Element 2 ICP-SF-MS) throughout Chapters Three to Five were performed under the conditions shown in Table 2.2. The criteria for the selection of the elements were based on their well-known importance in biological systems (Kozma and Ferke 1970; Cooper et al. 1978; Rath et al. 1980; Usdin et al. 1980; Leong et al. 1981; Atwood and Yearwood 2000) and their limits of detection associated with experimental conditions. For example, elements such as Al could not be measured with confidence since its detection limit is in the ng ml$^{-1}$ range. Furthermore, concentrations of Al in whole rat brain have been recorded in the order of 40 ng g$^{-1}$ (Gélinas et al. 1992) and as the rat will serve as the animal model throughout this work, Al was not measured. Internal standards were selected in order to reflect similar matrix-induced signal effects as the targeted analytes. Finally, it should be noted that the resolution settings given in Table 2.2 for the various elements were selected based on known spectral interferences (Jakubowski et al. 1998).
Table 2.2 Instrument parameters and measurement settings.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Setting</th>
</tr>
</thead>
<tbody>
<tr>
<td>RF power</td>
<td>1250 W</td>
</tr>
<tr>
<td>Cool gas</td>
<td>16 Lmin.⁻¹</td>
</tr>
<tr>
<td>Auxiliary gas</td>
<td>0.8 Lmin.⁻¹</td>
</tr>
<tr>
<td>Sample gas</td>
<td>0.9 Lmin.⁻¹</td>
</tr>
<tr>
<td>Additional gas</td>
<td>0.05 Lmin.⁻¹</td>
</tr>
<tr>
<td>Spray chamber</td>
<td>Glass Expansion® Scott type (quartz)</td>
</tr>
<tr>
<td>Nebuliser</td>
<td>Glass Expansion® Conikal (quartz)</td>
</tr>
<tr>
<td>Sample/skimmer cones</td>
<td>Nickel</td>
</tr>
<tr>
<td>Isotopes, (internal standards)</td>
<td>LRM – $^{107}$Ag, $^{111}$Cd, $^{202}$Hg, $^{208}$Pb, $^{209}$Bi, $^{238}$U, ($^{115}$In, $^{126}$Te)</td>
</tr>
<tr>
<td>and resolution mode (m/Δm)</td>
<td>MRM – $^{51}$V, $^{52}$Cr, $^{55}$Mn, $^{56}$Fe, $^{59}$Co, $^{60}$Ni, $^{63}$Cu, $^{66}$Zn, ($^{115}$In, $^{126}$Te)</td>
</tr>
<tr>
<td>Mass window</td>
<td>LR 150%, MR 200%, HR 200%</td>
</tr>
<tr>
<td>Search window</td>
<td>LR 80%, MR 60%, HR 60%</td>
</tr>
<tr>
<td>Integration window</td>
<td>LR 80%, MR 80%, HR 80%</td>
</tr>
<tr>
<td>Integration type</td>
<td>Average</td>
</tr>
<tr>
<td>Regression type</td>
<td>Linear</td>
</tr>
<tr>
<td>Replicates</td>
<td>Runs (∗3), Passes (∗3)</td>
</tr>
<tr>
<td>Sample time</td>
<td>LR 10 ms, MR 20 ms, HR 30 ms</td>
</tr>
<tr>
<td>Samples per peak</td>
<td>LR 10, MR 20, HR 30</td>
</tr>
<tr>
<td>Scan type</td>
<td>Electric scan</td>
</tr>
<tr>
<td>Detection mode</td>
<td>Analogue/Counting (“Both” mode)</td>
</tr>
</tbody>
</table>

In order to develop a quantitative analytical method, it is necessary to carry out a calibration of the elements to be examined using standard solutions. A sequence of the solutions to be analyzed is established with a blank (background intensity) and concentration calibration at the outset followed by the samples. Defined in the sequence are the parameters which are to be used for the measurements (e.g. method, tune file), concentrations of standards, and the quantification method to be used (e.g. standard addition, external calibration). With a sequence of analysis completed, the instrument is ready to acquire and evaluate data.

Each acquired raw data point consists of a data pair: the mass number (in mass units [u]) and the intensity (in counts per second [cps]). During scans, data are acquired as samples and intensities. In a bar graph spectrum (Figure 2.7) each sample acquired is
represented by a bar. Its position on the abscissa represents the mass at which the sample was taken and the ordinate represents the number of counts recorded during the sampling time. The sampling time is the distance between two neighboring sample bars. In order to acquire an accurate peak representation, at least 10 bars must be spread over the peak width to obtain a correct picture of the peak shape. On the other hand a sufficiently long sampling time must be selected to obtain enough counts per second for a proper statistical representation.

2.5 Analytical Performance of Thermo Fisher Element 2 ICP-SF-MS

2.5.1 Resolution

The separation of an interfering ion signal from that of an analyte is dependent upon the mass difference and the spectral resolution of the instrument. The mass resolution \( R \) is generally defined as:

\[
R = \frac{m}{\Delta m}
\]  

(2.3)

where \( \Delta m \) is the mass difference required to achieve a valley of 10% between two adjacent peaks (Jakubowski et al. 1998).

To demonstrate the resolution of ICP-SF-MS, a 10 ng ml\(^{-1}\) solution of Se (2% v/v nitric acid, Suprapur\textsuperscript{®}) was analyzed using the three resolution modes of the Element 2. Selenium is traditionally considered to be one of the most difficult elements to analyze by ICP-MS due to Ar based molecular ions interference and its relatively high
ionization energy (9.8 eV) causing poor ionization efficiency (ca 30%) in the plasma (ThermoFinniganMAT 2001).

Table 2.3 compares the theoretical isotope ratio of $^{77}$Se (normalized to $^{82}$Se) with those found experimentally in low, medium and high resolution mode. Comparing the isotope ratio gives a good indication of whether or not the isotope has been resolved from interference peaks. Figure 2.8 shows the low, medium and high resolution spectra of $^{77}$Se and its major interferent species $^{40}$Ar$^{37}$Cl. $^{77}$Se is partially resolved in medium resolution. Although its isotope ratio is significantly improved from that found in low resolution, medium resolution is still not suitable for accurate determination. Hence, high resolution is required for $^{77}$Se to be completely resolved from its primary interferent $^{40}$Ar$^{37}$Cl.

Table 2.3 Comparison of theoretical and experimental isotopic ratios of $^{77}$Se normalized to $^{82}$Se (a summary of twenty analyses) in low, medium and high resolution mode using the Element 2.

<table>
<thead>
<tr>
<th>Isotope</th>
<th>Theoretical %</th>
<th>Theoretical (Normalized)$^1$</th>
<th>Experimental LRM (Normalized)</th>
<th>Experimental MRM (Normalized)</th>
<th>Experimental HRM (Normalized)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Se77</td>
<td>7.60</td>
<td>82.6</td>
<td>934 ± 3</td>
<td>96.2 ± 0.7</td>
<td>82.7 ± 1.0</td>
</tr>
<tr>
<td>Se82</td>
<td>9.20</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

$^1$ Abundances are normalized by: ($^x$Se intensities / $^{82}$Se) × 100%.
Figure 2.7 Mass spectra of $^{77}\text{Se}$ in: (a) low; (b) medium; and (c) high resolution.
2.5.2 Limits of Detection

The limit of detection (LOD) is the lowest concentration of analyte that can be measured with reasonable certainty in a given analytical procedure. The LOD for ICP-MS is calculated using (ThermoFisher 1999):

\[
\text{LOD} = (3 \times \text{standard deviation of } n \text{ blank intensities } \times \text{concentration of standard}) \\
\text{(intensity of standard } - \text{ average intensity of blank)}
\]

(2.4)

where \( n \geq 10 \).

For the determination of the LOD, a series of 10 ng ml\(^{-1}\) multi-element standards (2% v/v nitric acid, Suprapur\(^\circledR\)) were prepared and analyzed using the parameters from Table 2.2. Additionally, ten blanks 2% nitric acid matrix was prepared and analyzed.

The detection limits of elements determined in this thesis are presented in Table 2.4.

Table 2.4 ICP-SF-MS instrument detection limits (IDL) expressed as pg ml\(^{-1}\) (3\(\sigma\), \(n = 10\)).

<table>
<thead>
<tr>
<th>Element</th>
<th>IDL</th>
<th>Element</th>
<th>IDL</th>
<th>Element</th>
<th>IDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ag</td>
<td>7</td>
<td>Cu</td>
<td>5</td>
<td>Ni</td>
<td>19</td>
</tr>
<tr>
<td>As</td>
<td>9</td>
<td>Fe</td>
<td>103</td>
<td>Pb</td>
<td>12</td>
</tr>
<tr>
<td>Bi</td>
<td>13</td>
<td>Hg</td>
<td>22</td>
<td>Se</td>
<td>97</td>
</tr>
<tr>
<td>Cd</td>
<td>3</td>
<td>In</td>
<td>4</td>
<td>U</td>
<td>8</td>
</tr>
<tr>
<td>Co</td>
<td>3</td>
<td>Mg</td>
<td>450</td>
<td>V</td>
<td>7</td>
</tr>
<tr>
<td>Cr</td>
<td>37</td>
<td>Mn</td>
<td>43</td>
<td>Zn</td>
<td>9</td>
</tr>
</tbody>
</table>
2.6 High Pressure Liquid Chromatography

Given the destructive nature of ICP to molecular species, a separation technique is required before ICP-MS determinations of metal species. The following gives an outline of the method used in this work along with the strategy for metalloprotein investigations.

The most often applied chromatographic technique for non-volatile compounds is HPLC (Heitkemper et al. 1989; Subramanian 1996) using various separation principles (e.g. reverse phase, ion exchange etc.). The technique HPLC involves the injection of a liquid sample (µL amount) into a column packed with porous silica-based or resin particles (stationary phase). Individual components of the sample are then transported through the column, containing the stationary phase material, by a liquid/solvent (mobile phase) and separated by physicochemical interactions with the particles of the stationary phase. After passing through the column, the separated components are then identified by an external measurement device, such as a diode array detector (DAD).

A HPLC system consists of a pump, injector, column and detector. The pump moves liquid/solvent (known as the mobile phase) through the column at a specified flow rate (usually in the range of 1-2 ml min$^{-1}$.) The injector introduces the liquid sample into the mobile phase and is usually integrated with an auto-sampler unit. The stationary phase of the column separates the sample components based on various parameters such as size, hydrophobicity and net charge. The DAD measures the UV-visible absorbance of the components eluting from the column and via the data system generates an output of the detector response known as a chromatogram. A DAD consists of a number of layered (and insulated from each other) photosensitive diodes.
A user-defined wavelength (or wavelength range) in the UV-visible spectrum, from a polychromatic source (usually a deuterium lamp), is directed through a flow cell and dispersed by a diffraction grating onto the surface of the DAD which in turn generates an electrical signal. Any eluting compounds absorbing this wavelength will reduce the amount of UV-visible energy reaching the DAD, resulting in a change in electrical signal, which is amplified and directed to a data system. A UV-visible spectrum can be acquired in order to identify the compound or class of compounds.

2.6.1 HPLC of metalloproteins

The primary consideration when designing a separation strategy for metalloproteins is the instability of the metal to protein binding. In order to minimize this, there are several requisites for metalloproteins separation including: 1) buffers (mobile phase) that must be either neutral or a weak base to avoid the dissociation of the protein-bound metal; 2) neither the buffers nor column material should bind to the metal component of the metalloproteins (Suzuki et al. 1983).

Ion exchange chromatographic (IEC) separations may be carried out at near physiological conditions which make them ideally suited to metalloproteins in order to avoid metal loss during extraction. Similarly, size exclusion chromatography (SEC) offers the advantage of maintaining the metalloprotein in its native form but suffers from relatively poor resolution (Regnier 1983). Both IEC and SEC routinely yield 80-100% recovery of biological activity (Regnier 1983).

IEC are based on electrostatic interactions between the mobile and stationary phases. These electrostatic interactions can involve either positive ions in the mobile phase binding to a negative charged stationary phase [known as cation exchange
chromatography (CEC)] or the reverse situation [known as anion exchange chromatography (AEC)]. CEC is not applied to metalloprotein separations due to the fact that they do not attach to this resin type at physiological pH. Typically, CEC is carried out between pH 4 and 7.

AEC separations are performed using a starting buffer, with a pH between 7 and 10. A second pH matched buffer solution containing 1M NaCl is introduced into the mobile phase and gradually increased in volume thereby creating a charge gradient between it and the starting buffer. The surface charge of the bound solutes will be net negative and will therefore compete with the chloride ions in the mobile phase for binding to the stationary phase. Commonly used anion exchange resins are Q-resin (a Quaternary amine) and DEAE resin (Figure 2.9). As the NaCl concentration increases the proteins will be released from their bound state at a given concentration depending on the strength of their net negative surface charge.

![Figure 2.8 Commonly used anion exchange resins.](image-url)
Proteins are retained by the interaction of its aspartic and glutamic amino acid side-chains, which have pK\(_a\) values of 3.65 and 4.25, respectively (Figure 2.10) (Nelson and Cox 2005), with the amine groups of the stationary phase resin. As the mobile phase is buffered at a pH greater than 4, retention of the proteins are largely dependent on the number of anionic side-chains present in them. Below pH 4 these acid side-chains begin to protonate and retention on the stationary phase decreases.

![Aspartic and glutamic acid side-chains](image)

Figure 2.9 Aspartic and glutamic acid side-chains.

### 2.7 Metalloprotein Screening Methodology

Metalloproteins can be characterized by analyzing the metal bound to the protein (after chromatographic separation), therefore a specific detector for metals (in the form of ICP-MS) would be ideal compared to a UV-visible detector (DAD) for a more unambiguous identification. As no single chromatographic method is capable of complete resolution of complex protein mixtures, multi-dimensional liquid chromatography (MDLC) separation methods are playing an important role in proteome research (Neverova and Eyk 2005; Tang et al. 2008). Similarly, metalloprotein studies will require MDLC but are limited to separation techniques that are not denaturing.
IEC together with reversed phase liquid chromatography (RPLC) (based on separation of hydrophobicity) has become an example of orthogonal two-dimensional LC analysis. Separation of molecules using IEC is based on charge interactions; whereas separation by RPLC relies on hydrophobic interactions. These techniques can offer improved purification of proteins when used in succession compared to when used individually. IEC is used as the first stage of protein separation due to its high salt matrix and incompatibility with protein mass spectrometry. The partially purified protein fractions are then loaded onto a RP column, which provides the final stage of separation and with being based on hydrophobicity, effectively removes salt from the separated protein fractions. The protein components are then analyzed using an ESI-MS tandem technique such as electrospray ionization quadrupole time-of-flight (ESI-Q-TOF) mass spectrometry. However, as RPLC is denaturing to the metalloproteins, any metal analysis/detection must be performed prior to this step. Figure 2.11 outlines the analytical methodology used in this thesis. After sampling and tissue preparation, samples are diverted through three streams, namely; 1) measurement of total metal content by ICP-SF-MS; 2) ICP-SF-MS measurement of metals after ultrafiltration separation; and 3) ICP-SF-MS measurement of metals and RPLC/ESI-Q-TOF analysis of protein components after AEC separation.
Figure 2.10 Methodology for metalloneurochemical analysis.

2.7.1 Tissue sampling

The animals were deeply anesthetized with an intra-peritoneal injection of 50 mg kg\(^{-1}\) ketamine and 5 mg kg\(^{-1}\) xylazine (Rhone Merieux, Australia). Sodium heparin (10 IU) (Astra Pharmaceuticals, Australia) was injected into the left ventricle of the heart and allowed to circulate for 90 s, whereupon the animal was perfused intra-cardially with 120 ml of cold 300 mM mannitol (phosphate buffered to pH 7.5; 320 mOsm). The brains were then carefully removed and stored at circa -80\(^\circ\)C for at least 24 hours. Brain regions were dissected in a laminar airflow cabinet. Whole brains were thawed in ice-cold mannitol and placed on a clean plastic tray, which rested on a bed of crushed ice. Cold mannitol was periodically flushed over the surface of the brain to prevent it from drying. No blood clots were observed. Plastic dissection tools (Proscitech, Australia) were used throughout the sampling procedure to prevent metal contamination of the samples. With respect to the sampling of the barrel cortex for metalloprotein investigation (presented in Chapters 4 and 5), the posteromedial barrel cortex (which represents the snout vibrissae) is readily delineated by its pattern of myelin deposition.
(Figure 2.11), and so transverse illumination with a bright light is sufficient to identify the boundaries of the barrels (Elston et al. 1997). A transfer pipette was used to extract the posteromedial barrel cortex region from the rest of the cortex. White matter was then removed from the extracted tissue.

Figure 2.11 Location and approximate dimensions of rat brain and barrel cortex regions.

2.7.2 Sample preparation of brain tissue

The sample preparation procedure for total metal ICP-SF-MS determination is described in chapter 3. With respect to extraction of MWCO <3 kDa and >3 kDa cytosol fractions from tissue (wet weight), the sample grinding kit (GE Healthcare) and Microcon ultracel YM-3 (Millipore) ultra-filtration units were employed. The extraction solution consisted of 20 mM Tris (Sigma) and 25 µM DTT (Sigma) pH adjusted to 7.5 with high purity HCl (30% v/v, Suprapur®, Merck). All solutions were prepared with polypropylene laboratory ware and using Milli-Q deionized water (18 MΩ cm at 25°C).
Ultra-filtrated extracts (100 µl) were diluted to 1 ml using 2% HNO₃ (diluted from high purity HNO₃ 65% v/v, Suprapur®, Merck) before ICP-SF-MS analysis.

2.7.3 AEC separation of proteins

HPLC was performed using an Agilent 1200 Series HPLC equipped with binary pump and DAD. Fractions were separated using a Biosep DEAE (7.8 x 75 mm) anion exchange column (Phenomenex). The optimized elution program consisted of an initial step at 100% A for 5 min, followed by two linear gradients from 5 to 20 min up to 50% B and from 20 to 24 min up to 100% B. Buffer A was 20 mM Tris (pH 7.5) with 25 µM DTT and buffer B was 20 mM Tris (pH 7.5) with 25 µM DTT and 1 M NaCl. The injection volume was 50 µL and the flow rate was a constant 1 ml min⁻¹. All connections were made with PEEK tubing (1/16” x 0.010”). Twenty-four 1 ml fractions were collected into 1.5 ml eppendorf tubes using a Foxy Jr. Fraction collector. The UV absorbance was monitored at 254 nm.

In order to validate the AEC method, a series of 50 µl injections of a 2.0 mg ml⁻¹ solution of carbonic anhydrase (Zn protein) were run under the above conditions. The dissolution of carbonic anhydrase (Sigma-Aldrich) was done in the starting buffer. Zn was analyzed using the parameters from Table 2.2. Presented in figure 2.12 is the spectrum of UV absorbance and Zn measured in the fractions collected followed by the calculation of Zn recovery from carbonic anhydrase (figure 2.13). Recoveries ranged from 97 to 105%.
From a stock solution of 2.0 mg ml$^{-1}$ carbonic anhydrase a 50 µl injection onto the DEAE column.

Therefore: $0.05 \text{ ml} \times 2.0 \text{ mg ml}^{-1} = 0.1 \text{ mg}$ carbonic anhydrase injected.

No. moles of carbonic anhydrase = $0.1 \times 10^{-3} \text{ g} \div 29000 \text{ g mol}^{-1} = 3.45 \times 10^{-9} \text{ mol}$

As there is a 1:1 mole ratio between Zn and protein ligand;

No. moles of Zn = $3.45 \times 10^{-9} \text{ mol} \times 65.37 \text{ g mol}^{-1} = 2.254 \times 10^{-7} \text{ g}$ (theoretical mass of Zn injected)

Collection of fraction 6 Zn concentration = 11.24 ng ml$^{-1}$

Percentage recovery of Zn = $11.24 \text{ ng ml}^{-1} \div 50 \mu l$ (injection volume)

= $2.248 \times 10^{-7} \text{ g} \div 2.254 \times 10^{-7} \text{ g} \times 100\%$

= 99.7% recovery of Zn

Figure 2.13 Working calculation of Zn recovery from injection of carbonic anhydrase (2.0 mg ml$^{-1}$) onto Biosep DEAE AEC column.
2.7.4 Protein Mass Spectrometry analysis

Samples were loaded onto the HPLC system (LC Packings Ultimate HPLC system, Dionex, Netherlands) with a 100 μm inside diameter pre-column of C18 reversed phase material (RPLC) (ReproSil-Pur 120 C18-AQ, 3 μm beads, Dr Maisch, Germany) in 0.1% formic acid 99.9% water. The peptides were eluted through a 50 μm internal diameter C18 column of the same material at a flow rate of 100 nl min$^{-1}$. The main part of the gradient was from 10% buffer B (90% acetonitrile, 0.1% formic acid and 9.9% water) to 65% buffer B in twenty eight minutes. The eluate was sprayed through a 10 μm i.d. distal coated SilicaTip (New Objective, USA) at 2000 V into a QSTAR XL Q-TOF MS (MDS Sciex/Applied Biosystems). Data dependent acquisition was done using a 1 s survey scan from which the three most abundant double, triple or quadruple charged peptides were selected for product-ion scans (2 s). The spectra were converted to peak lists using the script Mascot.dll version 16b23 within the Analysis QS 1.1 software (MDS Sciex). Database searching for proteins was performed using a local copy of Mascot version 2.2 (Matrix Science, London, UK). The searched database was SwissProt 56.6 (405506 sequences; 146166984 residues). The searches were done with the variable modifications: deamidation (NQ), oxidation (M). The precursor ion mass tolerance was 200 ppm and fragment ion tolerance was 0.1 Da. Enzyme specificity for tryptic digests was selected to trypsin with 1 missed cleavage. A cut-off score of 30 and significance threshold of p < 0.05 was considered acceptable for identification.
Chapter Three: Profiling of trace elements in healthy rat brain tissue

3.1 Introduction

Neurochemical studies of the CNS have generally centered on organic biomolecules, such as proteins and DNA (Burdette and Lippard 2003). In the process of these investigations the functions of metal ions such as Zn\(^{2+}\), Ca\(^{2+}\), Mg\(^{2+}\), K\(^+\) and Na\(^+\) have also been analyzed using traditional trace element detection techniques (Burdette and Lippard 2003). It is only with the advent of ICP-MS that quantitative trace element investigation of the CNS has become possible (due to the analysis being measured under conditions that allow direct comparison of elemental concentrations) and only a few such investigations have recently emerged (Fujimura et al. 1992; Gélinas et al. 1992; Gélinas and Schmit 1994; Saito et al. 1995). Most “reference values” for trace element content in the CNS of “normal” populations gather results from disparate studies performed by researchers using distinct techniques and a wide variety of instruments (Gélinas et al. 1992).

There is a clear need to focus on the quantification of essential and toxic elements in neurological tissues under nutritional, biomedical and environmental controlled conditions. Such investigations are required to elucidate the effects of these elements on the mammalian brain. Earlier studies have shown that many trace elements are delicately balanced and play a pivotal metabolic role (Cumings 1959; Cumings 1965; Kozma and Ferke 1970; Cooper et al. 1978; Rath et al. 1980; Usdin et al. 1980). Hence, if these balances are actively disturbed, such as from the ingestion or inhalation of high dosages over long periods of time, then they may become toxic. Due to the increasing heavy metal pollution experienced by our modern societies, neurological studies of trace elements is of paramount importance, since it will not only shed knowledge on the
appropriateness of the environment toward human and animals habitation, but also on possible causes for neurological disorders (Atwood and Yearwood 2000).

The mammalian brain is extremely complex and is a highly specialized organ of the body. Specific regions of the brain have been shown to have an unequal distribution of metabolic enzymes (Leong et al. 1981), neurotransmitter substances (Lai et al. 1981) and trace elements (Rajan et al. 1976; Kemp and Danscher 1979; Chan et al. 1983; Takahashi et al. 2001). In the case of the latter, a variety of elemental detection techniques have been employed to determine these concentrations. Typically the techniques used centered on AAS, PIXES, ICP-AES, and NAA. For example, Rajan et al. (Rajan et al. 1976) used AAS to analyze Cu, Fe, Zn, Mg and Ca in the synaptosomal, myelin and mitochondrial fractions of Sprague-Dawley rat brains. Also, Kemp et al. (Kemp and Danscher 1979) used PIXES to analyze thirteen elements including Zn, Rb, Fe, Pb and Ni with formalin “fixed” and “unfixed” samples of the hippocampus, neocortex, amygdala and spinal cord of male Wistar rat brains. Takahashi et al. (Takahashi et al. 2001) used ICP-AES to measure P, K, Na, Mg, Ca, Fe, Zn, Cu, Rb, Al, Mn, Sr, Mo, Co, Pb, Cs and Ca using the whole brain of Sprague-Dawley rats. Finally, Chan et al. (Chan et al. 1983) utilized NAA to simultaneously determine thirty elements in the brain regions of female Wistar rats (MCR Porton strain). Specifically this group concentrated on the hypothalamus, striatum, mid-brain, cerebral cortex, pons and medulla, hippocampus and cerebellum. Marked regional differences in elemental concentrations were observed, but no explanation or statistical analysis was given for these variations.

There have been only three previous investigations of tissue from specific brain regions of rats using ICP-MS. Two of these tissue investigations have centered on using ICP-Q-MS and the other employed ICP-SF-MS. Fujimura et al. (Fujimura et al. 1992)
measured Al, Sc, V, Cr, Mn, Cu, Rb, Sr and Mo in cerebellum, brain stem regions, hypothalamus, mid-brain combined with thalamus, striatum, hippocampus and cerebral cortex using male Wistar rats exposed to 1 to 3 periods of restraint conditions, namely 15, 30 and 60 minutes stress periods. Moreover, Saito et al. (Saito et al. 1995) measured Cu, Mg, Mo, Rb, and Zn in the same brain regions using Long-Evans Cinnamon and Long-Evans agouti rats. Lastly, Becker et al. (Becker et al. 2005b) utilized laser ablation ICP-SF-MS to measure Cu, Zn, P and S distribution in thin sections of the Caudatus putamen of a male F344 Fisher rat brain hemisphere for the detection of a small-sized tumor (thickness: 20 μm).

In this chapter, a general procedure was developed for the simultaneous ICP-SF-MS determination of some essential and toxic trace elements in specific brain regions of eight healthy male Wistar rats (Paul et al. 2004). In order to assess the relation of trace elements with different neural conditions, it is essential to have a baseline status of trace elements in the CNS in a healthy sample of the population. Therefore the purpose of this study was to determine reference values of trace elements in the brain of healthy subjects using the recently validated ICP-SF-MS method to study the effects of experience dependent plasticity on levels of trace elements.

The brain regions have been specifically chosen (e.g. olfactory bulb, telencephalon, pons and medulla, superior/inferior colliculus, diencephalon, hippocampus and cerebellum) because they are readily identified, thereby enabling consistent tissue extraction. This is in contrast to the two previous ICP-Q-MS studies (Fujimura et al. 1992; Saito et al. 1995), which have extracted tissue from combined regions of the brain (e.g. brain stem regions and mid-brain combined with thalamus). To our knowledge there have been no high-resolution studies of specific brain regions of Wistar rats as a benchmark (even though their genetic diversity is eliminated via in-
breeding). Furthermore, the most characteristic elemental pattern of each tissue in this study has been identified using a principal component analysis (PCA), which enabled identification of the correlations between elements and tissue investigated. No such analysis has been performed in the previous ICP-MS studies (Fujimura et al. 1992; Saito et al. 1995).

3.2 Experimental and Analytical Procedure

3.2.1 Laboratory ware, reference material, reagents and standards

All solutions were prepared with polypropylene laboratory ware [pipette tips (Rainin edp3™ electronic pipettes), volumetric flasks (Kartell) and sample tubes (Cetac)]. Standard solutions were prepared from Perkin Elmer 10 µg ml⁻¹ multi-element ICP-MS standards and 1000 µg ml⁻¹ single-element ICP-MS standards (In and Te). High purity HNO₃ (65% v/v, Suprapur®, Merck) and H₂O₂ (30% v/v, Suprapur®, Merck) were used as received. Reagent grades of mannitol (>98%, Sigma), Na₂HPO₄ and NaH₂PO₄ (98%, Sigma) were used as received. All solutions were prepared using MilliQ deionized water (18 MΩ·cm at 25°C). The NIST SRM 1577b bovine liver was used to validate the tissue sample preparation and analysis methodologies, since it is the most relevant SRM with respect to matrix and analyte concentrations in this investigation. It should be noted that there is no SRM available at present for brain tissue.
3.2.2 Sample preparation

The extracted tissues were weighed after they were added to the TFM microwave vessels. The masses of tissues ranged from 60 mg to 700 mg (wet weight). Four and 2 ml of 65 % (v/v) HNO$_3$ and 30 % (v/v) H$_2$O$_2$ were then added to the digestion vessels, respectively. The tissue was then microwave digested using a Milestone MEGA system and digestion program for organic samples (Milestone 1995). For biological tissues the latter consisted of five power stages: 250, 0, 250, 400 and 600 W for a duration of 1, 2, 5, 5 and 5 minutes, respectively. The digests were then diluted to 50 ml (2% v/v HNO$_3$), with a higher dilution of 100 ml employed for the higher concentration elements (i.e. Cu, Fe and Zn).

Aqueous standards (2% v/v HNO$_3$) covering the concentration range 0 – 50 ng ml$^{-1}$ were used for external calibration. Six calibration standards were employed to generate the calibration lines with each giving correlation coefficients of at least 0.999.

3.2.3 Sampling

Five male out-bred Wistar rats, weighing 400 – 450g and aged 20-22 weeks, were used in this study. These animals were bred at The University of Newcastle Central Animal Facility and were fed standard laboratory rat food (Gordon’s Specialty Stockfeeds, Australia). The animals were deeply anaesthetised with an intraperitoneal injection of 150 mg kg$^{-1}$ sodium pentabarbitone (Rhone Merieux, Australia). Sodium heparin (10 IU), (Astra Pharmaceuticals, Australia) was injected into the left ventricle of the heart and allowed to circulate for 90 seconds, whereupon the animal was perfused intracardially with 120 ml of cold mannitol (phosphate buffered to pH 7.5; 320 mOsm). The brains were then carefully removed and stored at –80° C for at least 24 hours.
Brain regions were dissected in a laminar airflow cabinet. Whole brains were thawed in ice-cold mannitol and placed on a clean plastic tray, which rested on a bed of crushed ice. Cold mannitol was periodically flushed over the surface of the brain to prevent it from drying. No blood clots were observed. Plastic forceps (Proscitech, Australia) were used throughout the sampling procedure in order to prevent metal contamination of the samples. Tissue was collected from the following brain regions: olfactory bulb, telencephalon (excluding basal ganglia and hippocampus), diencephalon (essentially thalamus and hypothalamus), hippocampus, inferior colliculus, superior colliculus, cerebellum, pons and medulla (Figure 3.1). Tissues were obtained bilaterally except for hippocampus and telencephalon where the right structure was analyzed by itself. Once removed, the dissected brain regions were stored in ice-cold mannitol, until ready for analysis.

Figure 3.1 Medial view of rat brain regions.

3.2.4 Method validation

The SRMs and spiked brain tissue (10 ng ml\(^{-1}\)) were prepared and analyzed as described above. To test instrumental performance, a post-digestion spike was added (10 ng ml\(^{-1}\)) to the sample. A SRM, pre-digestion spike and post-digestion spike were analyzed every eight samples, corresponding to the eight tissue regions of one rat. Table
3.1 shows the percentage recoveries of the SRM, pre-digestion and post-digestion spikes. The SRM and digested spikes gave excellent recoveries for all of the elements, with the exception of Cd, Pb, As and Se (in the SRM), which recorded slightly lower recoveries (i.e. 85 – 89%). Based on the full recoveries of the post-digestion spikes, no matrix effects were produced by the brain tissue samples.

Table 3.1 Percentage recoveries of SRM 1577b bovine liver and spikes at the 90% confidence limit ($n = 5$).

<table>
<thead>
<tr>
<th>Elements</th>
<th>SRM (%)</th>
<th>Brain tissue spiked (pre-digestion) (%)</th>
<th>Brain tissue spiked (post-digestion) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ag</td>
<td>115 ± 10</td>
<td>95 ± 10</td>
<td>110 ± 10</td>
</tr>
<tr>
<td>As</td>
<td>86 ± 6</td>
<td>105 ± 8</td>
<td>110 ± 10</td>
</tr>
<tr>
<td>Bi</td>
<td></td>
<td>106 ± 4</td>
<td>110 ± 5</td>
</tr>
<tr>
<td>Cd</td>
<td>89 ± 5</td>
<td>107 ± 6</td>
<td>90 ± 5</td>
</tr>
<tr>
<td>Co</td>
<td>104 ± 5</td>
<td>104 ± 5</td>
<td>105 ± 5</td>
</tr>
<tr>
<td>Cr</td>
<td></td>
<td>98 ± 5</td>
<td>110 ± 5</td>
</tr>
<tr>
<td>Cu</td>
<td>105 ± 5</td>
<td>105 ± 4</td>
<td>100 ± 4</td>
</tr>
<tr>
<td>Fe</td>
<td>107 ± 5</td>
<td>97 ± 5</td>
<td>105 ± 5</td>
</tr>
<tr>
<td>Hg</td>
<td>115 ± 10</td>
<td>112 ± 10</td>
<td>110 ± 10</td>
</tr>
<tr>
<td>Mn</td>
<td>98 ± 4</td>
<td>109 ± 5</td>
<td>102 ± 5</td>
</tr>
<tr>
<td>Ni</td>
<td></td>
<td>101 ± 5</td>
<td>110 ± 6</td>
</tr>
<tr>
<td>Pb</td>
<td>85 ± 5</td>
<td>105 ± 6</td>
<td>90 ± 5</td>
</tr>
<tr>
<td>Se</td>
<td>85 ± 6</td>
<td>113 ± 8</td>
<td>114 ± 10</td>
</tr>
<tr>
<td>U</td>
<td></td>
<td>104 ± 4</td>
<td>110 ± 5</td>
</tr>
<tr>
<td>V</td>
<td>115 ± 8</td>
<td>107 ± 10</td>
<td>118 ± 10</td>
</tr>
<tr>
<td>Zn</td>
<td>104 ± 5</td>
<td>98 ± 6</td>
<td>100 ± 5</td>
</tr>
</tbody>
</table>
3.2.5 Statistical analysis

Confidence limits were given by the mean value ± ts / √n, where s was the standard deviation and t was the t-table value at a 90% confidence level. The estimated uncertainties were based on the combined effects of the digestion method, imprecision/instabilities of the instrument and the variability of the biological tissues. In the latter case, eight healthy male Wistar rats were chosen in order to minimize the individual variability. Using SAS software (SAS 2003), PCA was performed to reveal any characteristic elemental patterns of the eight analyzed tissue regions.

3.3 Results

Sixteen trace elements were chosen, namely, Ag, As, Bi, Cd, Co, Cr, Cu, Fe, Hg, Mn, Ni, Pb, Se, U, V and Zn in order to give a thorough screening of the inorganic content of brain tissue. In total ca. 45 elements could have been screened. Elements such as C, N and O are not currently measurable using ICP-SF-MS, whereas the relatively high detection limits of P, S, Cl and Br (due to their high ionization energies) combined with the small amounts of tissue available for analysis precluded them from being measured routinely, with respect to this study. Although Ca, K, Mg and Na can be measured by ICP-SF-MS, these elements are more easily measured by AAS, because of their high concentrations in the rat brain tissues. These elements are the most common and have been well characterized in the literature.

The trace elements that were analyzed can be divided into two basic categories, namely: essential (V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Se) (i.e. those elements that have established physiological roles); and non-essential (Ag, Cd, Hg, Pb, Bi, U, As) (i.e. those elements that are toxic or have an unclear physiological role). Moreover, the
essential trace elements listed are also those that will be monitored in terms of experience-dependent plasticity experiments, which will be presented in the following chapters.

3.3.1 Tissue sampling

It should be noted that there is an extensive network of blood vessels throughout the brain. Thus, in order to accurately measure the elemental distribution of brain tissue, without the confounding influence of elements within the blood (in particular Cu, Fe and Zn), the blood needs to be effectively flushed out of the system. Thus, the technique of Gélinas et al. (Gélinas et al. 1992) was adapted to include standard intracardial perfusion, which involves deeply anaesthetizing animals, injecting an anticoagulant into the blood stream and, under slight pressure, replacing the circulating blood with another solution. In this study, the solution was an iso-osmotic concentration of buffered mannitol.

In order to reduce inter-sample variation, well-defined areas of the brain were chosen and animals of the same sex were used. Male rats were selected, as they are less likely to be influenced by periodic hormonal fluctuations. Additionally, animals were euthanased at approximately the same time every day in order to control for possible diurnal influences. As the regions chosen perform a variety of functions and are anatomically dissimilar, a distinct elemental pattern might be expected.

3.3.2 Trace element distribution in rat brain tissue

Table 3.2 summarizes the essential trace element distribution, averaged from five healthy male Wistar rats covering eight specific brain regions. These essential trace
elements can be sub-divided into a high concentration (Fe, Cu and Zn) and a low concentration (V, Cr, Mn, Co, Ni and Se) sub-sets. Figure 3.2 highlights the mean distribution of these two sub-sets in a three-dimensional plot.

As expected, Table 3.2 and Figure 3.2 clearly reflect the elemental distribution of some known biochemical characteristics of the analyzed tissue. The elements in high concentrations, namely Fe, Cu and Zn, exhibit the largest absolute variations between the individual animals across the eight brain regions. Nevertheless, in terms of relative errors, these fall within the boundaries of relative error across both sub-sets.

Of these elements, Cu has the lowest concentration in the eight regions of the brain investigated. It is evenly distributed across these regions. Also, Fe is uniformly distributed across the eight regions, with a slightly higher concentration in the cerebellum. Of the three, Zn shows the greatest variability, with its highest concentrations in cerebellum and telencephalon and its lowest concentration being in the inferior and superior colliculi. This in accord with Zn being an important trace element involved in a variety of functions including synaptic plasticity (Choi and Koh 1998).
Table 3.2 Mean essential trace element distribution (µg g⁻¹) of eight brain regions at the 90% confidence limit (n = 5).

<table>
<thead>
<tr>
<th>Elements</th>
<th>Olfactory bulb</th>
<th>Telencephalon</th>
<th>Superior colliculus</th>
<th>Pons and medulla</th>
</tr>
</thead>
<tbody>
<tr>
<td>Co</td>
<td>0.006 ± 0.002</td>
<td>ND</td>
<td>ND</td>
<td>0.036 ± 0.014</td>
</tr>
<tr>
<td>Cr</td>
<td>0.018 ± 0.006</td>
<td>ND</td>
<td>ND</td>
<td>0.023 ± 0.007</td>
</tr>
<tr>
<td>Cu</td>
<td>1.378 ± 0.289</td>
<td>1.445 ± 0.157</td>
<td>1.248 ± 0.302</td>
<td>1.392 ± 0.210</td>
</tr>
<tr>
<td>Fe</td>
<td>7.558 ± 0.449</td>
<td>7.371 ± 0.409</td>
<td>6.704 ± 0.562</td>
<td>7.393 ± 0.303</td>
</tr>
<tr>
<td>Mn</td>
<td>0.196 ± 0.060</td>
<td>0.261 ± 0.078</td>
<td>0.274 ± 0.063</td>
<td>0.333 ± 0.033</td>
</tr>
<tr>
<td>Ni</td>
<td>0.985 ± 0.245</td>
<td>0.166 ± 0.019</td>
<td>0.528 ± 0.060</td>
<td>2.076 ± 0.631</td>
</tr>
<tr>
<td>Se</td>
<td>0.191 ± 0.065</td>
<td>0.090 ± 0.017</td>
<td>0.256 ± 0.137</td>
<td>0.158 ± 0.027</td>
</tr>
<tr>
<td>V</td>
<td>0.018 ± 0.002</td>
<td>ND</td>
<td>ND</td>
<td>0.011 ± 0.001</td>
</tr>
<tr>
<td>Zn</td>
<td>5.823 ± 0.459</td>
<td>7.708 ± 0.504</td>
<td>4.399 ± 0.509</td>
<td>4.896 ± 0.478</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Elements</th>
<th>Inferior colliculus</th>
<th>Diencephalon</th>
<th>Hippocampus</th>
<th>Cerebellum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Co</td>
<td>0.006 ± 0.002</td>
<td>0.029 ± 0.009</td>
<td>0.002 ± 0.001</td>
<td></td>
</tr>
<tr>
<td>Cr</td>
<td>ND</td>
<td>0.002 ± 0.001</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Cu</td>
<td>1.218 ± 0.324</td>
<td>1.291 ± 0.194</td>
<td>1.151 ± 0.241</td>
<td>1.654 ± 0.164</td>
</tr>
<tr>
<td>Fe</td>
<td>6.727 ± 0.846</td>
<td>7.140 ± 0.812</td>
<td>5.613 ± 0.540</td>
<td>9.348 ± 0.728</td>
</tr>
<tr>
<td>Mn</td>
<td>0.262 ± 0.070</td>
<td>0.270 ± 0.063</td>
<td>0.242 ± 0.053</td>
<td>0.295 ± 0.047</td>
</tr>
<tr>
<td>Ni</td>
<td>1.335 ± 0.112</td>
<td>0.426 ± 0.132</td>
<td>1.087 ± 0.390</td>
<td>0.281 ± 0.079</td>
</tr>
<tr>
<td>Se</td>
<td>0.384 ± 0.055</td>
<td>0.143 ± 0.037</td>
<td>0.241 ± 0.091</td>
<td>0.117 ± 0.041</td>
</tr>
<tr>
<td>V</td>
<td>0.019 ± 0.008</td>
<td>0.002 ± 0.001</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Zn</td>
<td>3.715 ± 0.706</td>
<td>5.900 ± 0.808</td>
<td>6.995 ± 1.735</td>
<td>7.921 ± 1.238</td>
</tr>
</tbody>
</table>

ND = not detected.
Rajan et al. (Rajan et al. 1976) also measured Cu, Fe and Zn using AAS in male Sprague-Dawley rats. The regions of brain tissue, which correspond to this study, are pons and medulla and cerebellum. The concentrations of Fe, Cu and Zn were several orders of magnitude higher (*i.e.* parts per thousand) since no perfusion was performed and these concentrations were expressed in terms of the metal ion $\mu$g mg$^{-1}$ of protein. Chan et al. (Chan et al. 1983) used NAA to determine Cu, Fe and Zn concentrations in female Wistar rats. The regions overlapping with our study were cerebellum, pons and medulla and hippocampus.
Figure 3.2 In eight brain regions, the mean distribution of: (a) essential high concentration trace elements; (b) essential low concentration trace elements.
They freeze dried their tissue and so measured the concentrations as dry weight. Consequently, their analysis included tissue and blood and so their concentrations were significantly higher (in µg g\(^{-1}\)) than our study, but significantly lower than the analysis of Rajan et al. (Rajan et al. 1976). Lastly, Gélinas et al. (Gélinas et al. 1992) measured the whole brain concentrations using ICP-Q-MS in male Sprague-Dawley rats. These workers used a perfusion technique, but they extracted the organs and then perfused with a syringe, whereas in the method outlined in this work, the perfusion was performed intracardially, thereby ensuring all blood was removed from the animal. Nevertheless, their concentrations for Cu, Fe and Zn in the whole brain were 2.334, 35.368 and 9.725 µg g\(^{-1}\), whereas mean concentrations over the eight regions were 1.347, 7.323 and 5.920 µg g\(^{-1}\), respectively. Only the comparative results for Cu and Fe are meaningful since these elements are relatively even distributed over the eight brain regions, which have been studied in this work. Of the two, only the Fe concentration varies significantly between the two studies. This may be attributed to the possible presence of residual blood in the Gélinas et al. (Gélinas et al. 1992) study due to their perfusion technique. For example, the measurement of Fe in erythrocyte and plasma by Gélinas et al. (Gélinas et al. 1992) was found to be 862.3 and 2.95 µg g\(^{-1}\), respectively. The perfusion technique outlined in this work has been well documented in the physiological literature and is a standard method in anatomical studies of the brain.

Table 3.2 and Figure 3.2 show the essential trace elements in low concentrations. The sub-set was dominated by three elements, namely, Mn, Ni and Se. It should be noted that Ni varies significantly in concentration over eight brain regions with it being mainly concentrated in the pons and medulla. In the case of Mn and Se, this study showed that these elements were evenly distributed over the eight brain regions and so can be compared to the study of Gélinas et al. (Gélinas et al. 1992). The latter whole
brain concentrations for Mn and Se were 0.279 and 0.104 µg g⁻¹, which compares favourably with the mean concentrations of 0.267 and 0.198 µg g⁻¹ given in this work, respectively.

Table 3.3 summarizes the non-essential trace element distribution, averaged from five healthy male Wistar rats, covering eight specific brain regions. Figure 3.3 highlights the mean distribution of these elements in a three-dimensional plot.

In the eight brain regions studied, Cd was below the detection limit whereas Hg was not detected in the telencephalon, superior colliculus, hippocampus and cerebellum. With the exception of Cd, all the non-essential elements were elevated in the inferior colliculus, and so were not uniformly distributed.
Table 3.3 Mean non-essential trace element distribution ($\mu$g g$^{-1}$) of eight brain regions at the 90% confidence limit ($n = 5$).

<table>
<thead>
<tr>
<th>Elements</th>
<th>Olfactory bulb</th>
<th>Telencephalon</th>
<th>Superior colliculus</th>
<th>Pons and medulla</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ag</td>
<td>0.174 ± 0.057</td>
<td>0.089 ± 0.022</td>
<td>0.315 ± 0.049</td>
<td>0.235 ± 0.069</td>
</tr>
<tr>
<td>As</td>
<td>0.154 ± 0.053</td>
<td>0.059 ± 0.007</td>
<td>0.217 ± 0.042</td>
<td>0.036 ± 0.008</td>
</tr>
<tr>
<td>Bi</td>
<td>0.345 ± 0.069</td>
<td>0.090 ± 0.009</td>
<td>0.439 ± 0.059</td>
<td>0.255 ± 0.058</td>
</tr>
<tr>
<td>Cd</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Hg</td>
<td>0.137 ± 0.012</td>
<td>ND</td>
<td>ND</td>
<td>0.551 ± 0.083</td>
</tr>
<tr>
<td>Pb</td>
<td>0.293 ± 0.063</td>
<td>0.207 ± 0.063</td>
<td>0.476 ± 0.150</td>
<td>0.402 ± 0.083</td>
</tr>
<tr>
<td>U</td>
<td>0.234 ± 0.041</td>
<td>0.055 ± 0.011</td>
<td>0.251 ± 0.041</td>
<td>0.193 ± 0.015</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Elements</th>
<th>Inferior colliculus</th>
<th>Diencephalon</th>
<th>Hippocampus</th>
<th>Cerebellum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ag</td>
<td>0.474 ± 0.069</td>
<td>0.117 ± 0.030</td>
<td>0.424 ± 0.077</td>
<td>0.067 ± 0.014</td>
</tr>
<tr>
<td>As</td>
<td>0.349 ± 0.122</td>
<td>0.079 ± 0.038</td>
<td>0.198 ± 0.085</td>
<td>0.061 ± 0.024</td>
</tr>
<tr>
<td>Bi</td>
<td>0.713 ± 0.148</td>
<td>0.185 ± 0.042</td>
<td>0.532 ± 0.117</td>
<td>0.120 ± 0.021</td>
</tr>
<tr>
<td>Cd</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Hg</td>
<td>1.043 ± 0.338</td>
<td>0.285 ± 0.074</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Pb</td>
<td>0.627 ± 0.048</td>
<td>0.140 ± 0.033</td>
<td>0.439 ± 0.094</td>
<td>0.096 ± 0.032</td>
</tr>
<tr>
<td>U</td>
<td>0.599 ± 0.194</td>
<td>0.119 ± 0.015</td>
<td>0.265 ± 0.128</td>
<td>0.074 ± 0.008</td>
</tr>
</tbody>
</table>

ND = not detected.

The inferior colliculus is the most metabolically active area of the brain, (Sokoloff et al. 1977) with the highest blood flow levels (Landau et al. 1955), therefore it is not surprising that the non-essential elements are preferentially distributed in this region of the brain.
3.4 Discussion

A multivariate approach was taken to highlight any characteristic element patterns within the different tissue regions. Principal component analysis (PCA) was employed to transform a relatively complex dataset into a simpler set of variables known as principal components. These principal components are ordered so that the first few components retain most of the variation present in all of the original variables (Jolliffe 2002).

Figure 3.4 (a)-(f) gives the existing relationships between the elements and rat brain tissues as generated by the PCA using four principal components. It highlights
80% of the corresponding multi-dimensional correlation structure. The variance between the inorganic contents of the five samples using a single organ (e.g., brain) would be expected to be lower than what would be expected between any pair of tissue from different organs (Gélinas et al. 1992).

Figures 3.4(a), (b) and (c) represents 61, 53 and 51% of the variability. In order to enable a simpler representation of these Figures, the olfactory bulb tissue and the essential trace elements of (V, Cr, Mn, Co) have been omitted, which were in low concentrations (see below).

The PCA showed that the olfactory bulb is at the origin of the axis in Figures 3.4(a)-(c), thereby indicating that it does not exhibit any characteristic elemental pattern and so was omitted from the figures for clarity. Figures 3.4(a)-(f) do show that the essential elements Fe and Cu are uniformly distributed and so are not characteristic or dominate in any of the eight brain regions (with Fe having a weak association to cerebellum). On the other hand, as demonstrated by Figures 3.4(a)-(d), Zn has a strong association with cerebellum and to a lesser extent with telencephalon.

The other essential elements (V, Cr, Mn, Co, Ni and Se) exhibit the following in the PCA: V and Cr have low concentration across the four and three brain regions, respectively and so show no associations (PCA not shown here); Mn is uniformly distributed across the eight brain regions and so is not characteristic of any one tissue (PCA not shown here); Co is measured in only six brain regions and is in very low concentrations showing little associations (PCA not shown here); Figures 3.4(a)-(c) show that Ni is most strongly associated with pons and medulla and hippocampus, and finally; Figures 3.4(a)-(c) show Se has the strongest association with the inferior colliculus and then to a lesser extent with superior colliculus and hippocampus. It has been shown that Se can reduce the toxicity of various transition metals (in particular
Hg) (Sasakura and Suzuki 1998). Hence, its higher concentration in the inferior colliculus, where there is a greater array of non-essential elements, may be of toxicological significance.

It is clear from Figure 3.4 that trace elements (As, Ag, Hg, Pb, U) are characteristic elements with respect to the inferior colliculus and of these, Pb is the most characteristic element in this region. The PCA demonstrates this and so armed with this information; on reviewing Table 3.3 it is now more readily evident that the concentration of Pb shows the greatest variation for this tissue when compared to the other non-essential elements. For example, to stress this point, Figures 3.4(a), (c), (e) and (f) show that Hg correlates strongly with the inferior colliculus, but also with pons and medulla.

3.5 Conclusion

The investigation of the brain tissue of healthy Wistar rats has now been established using ICP-SF-MS. Tissue concentrations of Ag, Cd, Hg, Pb, Bi, U, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As and Se in the olfactory bulb, telencephalon, pons and medulla, superior/inferior colliculus, diencephalon, hippocampus and cerebellum brain regions have been investigated. This study showed that the olfactory bulb does not exhibit any characteristic elemental pattern. The essential elements Fe and Cu are uniformly distributed and so are not characteristic of the eight brain regions under investigation. On the other hand, Zn is shown to have a strong association with the cerebellum and to a lesser extent with the telencephalon. The elements V and Cr have low concentrations across the four and three brain regions, respectively. Manganese is uniformly distributed across the eight brain regions and so is not characteristic of any one tissue, whereas Co is detected in only six brain regions and is in very low
concentrations showing little associations. Nickel is strongly associated with the pons and medulla and hippocampus, and Se has the strongest association with the inferior colliculus and then to a lesser extent with superior colliculus and hippocampus. The trace elements (As, Ag, Hg, Pb, U) are associated with respect to the inferior colliculus and of these, Pb is the most characteristic element in this region, whereas Hg correlates strongly with the inferior colliculus, but also with pons and medulla.
Figure 3.4 Individual loadings of elements and tissues on the four principal components (representing 80% of the variability) generated by the PCA where: (a) PC1 – PC2; (b) PC1 – PC3; (c) PC1 – PC4; (d) PC2 – PC3; (e) PC2 – PC4; (f) PC3 – PC4.
Chapter Four: Measurement of protein-bound and free zinc fluctuation in rat cerebral cortex following reduced sensory input by vibrissae removal

4.1 Introduction

The representation of the vibrissae in the rodent cortex, the barrel cortex, has several advantages for the study of learning- or experience-dependent changes in the cerebral cortex. The principal advantage being its unique cytoarchitecture (Kossut 1992): the mystacial vibrissae present on the rodent snout are arranged in five rows (A-E) and each vibrissa projects to a discrete collection of neurons (known as barrels) present in layer IV of the contralateral somatosensory cortex (Woolsey and Loos 1970). This allows both the straightforward study of the system using anatomical or physiological techniques and the mapping of neural modifications. Moreover, the barrel system’s peripheral sensory input can be easily manipulated, by stimulation (Takeda et al. 1999; Brown and Dyck 2005), denervation (Czupryn and Skangiel-Kramska 2001a), or trimming/removal of the vibrissae (Land and Akhtar 1999; Quaye et al. 1999). Consequent changes in the barrel cortex include short- and long-term changes in neural response profiles (Fox 2002) and long term terminal sprouting of axons of across barrel projections (Kossut 1992) and of dendritic growth of barrel neurons (Tailby et al. 2005).

Several studies have examined zinc levels in the barrel cortex of rodents after whisker manipulation using histochemical staining techniques. Typically, sensory deprivation by trimming, or plucking, selected vibrissae results in an increase in synaptic zinc staining in the corresponding deprived barrels as compared to neighbouring non-deprived barrels. There appears to be a developmental critical period
wherein sensory deprivation by vibrissal removal or trimming leads to a permanent change in synaptic zinc (Land and Akhtar 1999; Quaye et al. 1999; Czupryn and Skangiel-Kramska 2001b; Czupryn and Skangiel-Kramska 2001a; Brown and Dyck 2003). Similar manipulation in adults leads to acute changes lasting various periods after vibrissae regrowth (Land and Akhtar 1999; Brown and Dyck 2002). Using the same staining technique a transient decrease in synaptic Zn in affected barrels has been shown with increased whisker stimulation and tactile learning (Brown and Dyck 2005). While these changes can be linked to known roles that Zn ions play in modulating synaptic transmission (Takeda 2000), the technique of histochemical staining for trace metals is limited in terms of quantitative interpretation by low sensitivity and non-specificity (Sumi and Suzuki 2002; Morawski et al. 2005). Moreover, the technique is limited to revealing free ions, whereas most metals in these systems exist in the form of metal-protein and/or metal-sugar complexes (unbound metallic ions are present at very low concentrations) and therefore are “invisible” to histochemical staining (Lammers et al. 1994; Bandwar et al. 1996). Approximately 90% of the total brain Zn is found in the form of metalloproteins (Frederickson 1989) where it acts either as a component of the catalytic site of enzymes or in a structural capacity (Cuajungco 1997).

Several publications have recently emerged utilizing ICP-MS instrumentation for examining brain tissue (Prange et al. 2001; Panayi et al. 2002; Srivastava and Jain 2002; Gellein et al. 2003; Becker et al. 2004; Paul et al. 2004; Becker et al. 2005c; Becker et al. 2005d; Becker et al. 2007). ICP-MS (in particular ICP-SF-MS) allows for the simultaneous determination of elements at trace concentrations (pg g⁻¹) using only small quantities (mg) of sample (Jakubowski et al. 1998; Stuewer and Jakubowski 1998; Marchante-Gayón et al. 1999).
There are very few quantitative data concerning the distribution of zinc during neural re-organization because of the difficulties of reliably measuring very low concentrations of these elements in small brain samples (Brown and Dyck 2005) which are now alleviated by the ICP-SF-MS approach. In order to correlate changes in zinc concentration with expected changes in neural activity and plasticity we used a well-studied manipulation of the whisker-to-barrel cortex pathway in young adult rats. Whiskers were plucked in a checker-board fashion which has been shown to induce plasticity over a wide area of the barrel cortex (Wallace and Fox 1999) and the entire area was sampled for ICP-SF-MS analysis.

4.2 Experimental and Analytical Procedure

4.2.1 Animals

The experiments were performed on 55 male out-bred Wistar rats aged 8-12 weeks. These animals were bred at The University of Newcastle Central Animal Facility and these studies were carried out with the approval of the Animal Care and Ethics Committee of The University of Newcastle.

4.2.2 Vibrissae removal

Vibrissae were plucked from the left side of the snout in a checker-board pattern whilst the animal was anesthetized using 1.5% isofluorane in oxygen. The rats were then left to recover for a period ranging from one hour to eight days. At the respective time point, tissue was extracted from the left and right barrel cortices and stored at -80°C in mannitol. A 24 h sham treatment group was also sampled in order to detect any non-specific effects related to the anesthesia and handling of the animals. These animals
were exposed to the same conditions as the treatment groups with the exception of the vibrissectomy.

4.2.3 Sample preparation and analysis

Preparation of samples and measurement of Zn have already been described in sections 2.4.3, 2.7.2 and 3.2.2.

4.2.4 Statistics

The statistical significance of the ICP-SF-MS results was estimated by using one-way analysis of variance (ANOVA), with Tukey (HSD for unequal n) post-hoc comparisons test (Statistica 7, StatSoft Inc.).

4.3 Results

Changes in Zn concentration induced by the unilateral checkerboard removal of snout vibrissae were matched in the barrel cortex of both hemispheres. Throughout this study there was no difference in Zn concentration, within one standard deviation, between the barrel cortex contralateral and ipsilateral to the checkerboard vibrissectomy at any of the control, sham or recovery time points (figure 4.1). Base level total Zn concentration was around 6 µg g⁻¹ in controls and experimental animals. In terms of changes in total Zn concentration, at 1 h after vibrissectomy, Zn increased to 7.5 and 6.5 µg g⁻¹ (from basal levels of 6.0 and 5.8 µg g⁻¹) in the contralateral- and ipsilateral-cortex, respectively.
Figure 4.1 Total Zn concentrations in barrel cortex contralateral and ipsilateral to checkerboard vibrissectomy. The results represent mean values ± SD [Control (n = 8), 1 hour (n = 5), 8 hours (n = 4), 24 hours (n = 7), 24 hours sham (n = 5), 96 hours (n = 5)]. Significantly different time points from control are marked ($P < 0.05^*, 0.01^{**}, 0.001^{***}$).

Conversely, a decrease to 4.5 and 3.4 µg g$^{-1}$ at 8 h was recorded in the contralateral- and ipsilateral-cortex, respectively. 24 h after vibrissectomy the concentration of Zn showed an increase to around 9 µg g$^{-1}$ in both contralateral and ipsilateral barrel fields. This significant increase was maintained at 96 h.

Low molecular weight and protein bound Zn were distinguished by separation at 3 kDa and examined in controls and at 8 and 24 h after manipulation (figures 4.2 & 4.3). As expected, the concentrations of low molecular weight Zn were substantially lower than the protein bound form – accounting for around 10% of the total Zn measured. Analysis of changes in the two components revealed that the vibrissectomy-induced
changes in total Zn concentration appear to result from changes in the low molecular weight component alone.

Figure 4.2 Low molecular weight Zn concentrations (MWCO < 3 kDa) in barrel cortex contralateral and ipsilateral to checkerboard vibrissectomy. The results represent mean values ± SD [Control (n = 5), 8 hours (n = 5), 24 hours (n = 5)]. Significantly different time points from control are marked ($P < 0.05^*, 0.01^{**}$).
Figure 4.3 High molecular weight Zn concentrations (MWCO > 3 kDa) in barrel cortex contralateral and ipsilateral to checkerboard vibrissectomy. The results represent mean values ± SD [Control (n = 5), 8 hours (n = 5), 24 hours (n = 5)], there were no significant differences at any time point.

4.4 Discussion

In the barrel-field representation of the snout vibrissae contralateral to a checkerboard vibrissectomy, significant changes in total Zn concentration were found. An initial increase in Zn one hour after the procedure was followed by a decrease (at 8 h) and then by a significant increase (above control levels) for the duration of the experiment (24 – 96 h). Increases in Zn concentrations following similar procedures have been reported previously in studies using histochemical staining for the metal (Czupryn and Skangiel-Kramska 2001b; Brown and Dyck 2002). However the decreased Zn concentration 8 h after vibrissectomy has not been previously reported. This finding has been confirmed through repeated independent experiments in which low- and high-molecular weight Zn containing fractions were separated. A substantial
reduction in Zn was found in the <3 kDa fraction at 8 h post vibrissectomy followed by a large increase at 24 h. Discrepancies between the sum of cytosolic (low- and high-molecular weight fractions) and total zinc were noted and may be attributed to a combination of the normal variation between two independent experiments, two different groups of animals and sample preparation techniques (i.e. extraction and acidification versus total digestion).

Zinc concentration changes in barrel-field cortex ipsilateral to the vibrissectomy showed the same pattern, and extent, as in contralateral cortex. However there was greater variability in the measurements of ipsilateral cortex Zn concentrations and some changes were not significantly different to controls.

Concomitant changes in somatosensory cortex of both hemispheres following vibrissectomy are consistent with physiological studies following peripheral denervation. Experiments in macaque monkey, marmoset and cat have shown that the short-term effects of sensory nerve denervation, or local anaesthesia, on the responses of cortical neurons are mirrored in contralateral and ipsilateral primary somatosensory cortex (Calford and Tweedale 1990; Clarey et al. 1996).

Table 4.1 shows the comparison between this study and the conventional Zn staining literature, represented as a percentage difference of Zn in the deprived area from control. It should be emphasized that total Zn measurements take into account protein-bound Zn as well as low-molecular weight forms such as cysteine- or histidine-bound Zn. Furthermore, the notion that Zn is available as a “free” ion is erroneous (Maret 2000). It has been determined experimentally that the concentration of “free” Zn ions (Zn$^{2+}$) would be as low as the picomolar level and are primarily strongly bound to proteins (Peck and Ray 1971; Atar et al. 1995). Therefore the analyses of < 3 kDa cytosolic fractions are comparable with those of the Zn staining literature.
Table 1 Comparison of Zn measurements with zinc staining values (% difference of zinc in the deprived area from control). Zn staining is expressed as: Relative Optical Density = deprived (mean of rows BD) / non-deprived (mean of row C); these are compared to changes measured contralateral to checkerboard vibrissectomy relative to controls in this study.

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NS = Not significantly different from control

The results of this study are consistent with the literature in that significant increases in low molecular weight Zn concentrations are observed for a period up to 96
h after vibrissectomy. Brown and Dyck (Brown and Dyck 2002) monitored additional time points at 14 d and 21 d where Zn was seen to return to basal levels. Higher percentage differences of 25 to 67% were recorded compared to those in the literature of 6 to 20%. This is attributed to the substantially higher sensitivity of ICP-SF-MS compared to histochemical staining and other limitations ascribed to the latter technique pointed out earlier (Lammers et al. 1994; Bandwar et al. 1996; Sumi and Suzuki 2002; Morawski et al. 2005). With respect to the aligned changes observed between the contralateral and ipsilateral cortex in this study, no such changes were observed in the Zn staining literature (Czupryn et al. 2001; Brown and Dyck 2002). The measurement of Zn in the entire barrel cortex as opposed to the more localized zinc staining analysis of individual rows of deprived and non-deprived barrels may explain why the former method recorded no net difference between the contralateral and ipsilaterial cortex.

Interestingly, this study shows a decrease in Zn concentration at 8 h, which is not consistent with the histochemical staining literature. Low molecular weight bound Zn (or “free” Zn) is believed to be held in pre-synaptic vesicles in glutamatergic neurons (Assaf and Chung 1984; Budde et al. 1997; Czupryn and Skangiel-Kramska 1997; Land and Akhtar 1999) and if there is a significant release of Zn, then in the absence of increasing the pool of Zn bound in proteins, which these results show is not the case, or recovered in some re-uptake mechanism, there could be a net loss concomitant with an increase in activity. The effect of selective loss of sensory input from the area of the receptive field of neurons in somatosensory cortex is a massive initial expansion of the receptive area (into adjacent unaffected regions) followed by a shrinking of the receptive field (Calford and Tweedale 1988). This is interpreted as an initial disruption of local inhibitory circuits (disinhibition) followed by a phase in which the local excitatory / inhibitory circuitry is rebalanced to result in a new small receptive
field. Similar events and interpretation have been described for auditory (Calford et al. 1993) and visual cortex response to selective partial sensory loss (Gilbert and Wiesel 1992; Schmid et al. 1995; Calford et al. 1999). Whereas the role of Zn in excitatory synapses is not fully understood (Frederickson et al. 2005), an initial disinhibition response to vibrissectomy would be expected to increase activity and release vesicular Zn – resulting in a reduced concentration. Brown and Dyck (Brown and Dyck 2005), using histochemical staining, found a decrease in Zn with a whisker stimulation paradigm (Table 4.1). An explanation for the source of the subsequent increase in Zn concentration is less evident. The most parsimonious explanation being a release of bound Zn from metallothionein stores probably mobilized by nitric oxide triggered by glutamate synapse over-activation (Frederickson and Bush 2001; Frederickson et al. 2004). This putative event is not necessarily inconsistent with our failure to find a change in bound Zn concentration as the metallothioneins have the capacity exchange Zn with proteins and to sequester Zn from cytosolic transportation mechanisms.

It is presumed that membrane transport proteins can reduce total and “free” Zn, although their nature and physiological roles are not known (Qin et al. 2008). As Zn levels were not seen to fluctuate significantly in the protein fraction, then these transport proteins may be weakly bound to Zn or have a more indirect role in Zn efflux. The dynamics of Zn binding and release from its strong binding sites in proteins and its transfer from one site to another are unknown (Maret 2000). While brain uptake mechanisms for some metals have been identified, metal efflux from the brain has received little attention (Miu et al. 2006).

As pointed out by Cravatt and Sorensen, most proteomics experiments have relied on general protein-staining methods for quantification (Cravatt and Sorensen 2000). Measurement of proteins by Coomassie blue lacks sensitivity and silver staining
displays protein-dependent differences in staining intensity. Both stains exhibit poor
dynamic range and should be considered semi-quantitative (Cravatt and Sorensen
2000).

Alternatively, the high sensitivity and selectivity of ICP-SF-MS make it an
efficient screening tool for identifying metalloproteins and fully quantifying changes in
their concentration. The method developed here combined with off-line HPLC
separation of the protein fraction can identify Zn species by their “metal signature” in
the CNS and; combined with molecular mass spectrometry techniques, characterize the
proteins to which Zn is associated thereby offering some insight into its biochemistry.

4.5 Conclusion

The fluctuation of Zn was investigated in the barrel cortex of rats contralateral and
ipsilateral to partial vibrissae removal (checkerboard pattern plucking). Quantitative
analysis was carried out using ICP-SF-MS. This allowed measurement of total Zn
content and separated low molecular weight (free) and protein bound components. A
significant decrease of Zn at 8 h after whisker plucking was revealed in both deprived
and non-deprived barrel cortices. Thereafter, zinc content increased above control levels
for the length of the study (up to 96 h), confirming previous studies using histochemical
staining methods for free zinc. All changes are attributable to free Zn as no change was
observed in the protein bound form as sampled at 8 and 24 hr post manipulation.
Chapter Five: Measurement of protein-bound and free metal ion fluctuation in rat cerebral cortex following reduced sensory input by vibrissae removal

5.1 Introduction

The whisker-to-barrel neuronal pathway of rodents has become a model system for investigations of neuronal plasticity and over the past decade has provided much information on the subject. This may be attributed to this regions distinctive segregated clusters of neurons (termed barrels) being easily identifiable both structurally and functionally. In addition, the barrel cortex is one of the few regions of the cortex lending itself to examination from birth through to adulthood.

Previous studies of the vibrissae manipulation paradigm that have examined the effects on metal concentration have focused only on Zn. Given the multi-element capability of ICP-SF-MS, a range of metals may be measured simultaneously allowing a more holistic approach to CNS investigations. This study utilizes this capability and presents additional data on the fluctuation of metalloproteins and unbound metals in the barrel cortex after vibrissae removal. Only data for those metals relatively higher in concentration are presented, namely, Fe, Cu, Mn and Mg. All of the metals screened in chapter three were measured in this study; however, their concentration levels were too low (< 0.1 µg g$^{-1}$) to reliably monitor any statistically significant change.

Metalloprotein studies of the CNS have been largely centered on their determination in CSF (Gellein et al. 2007; Michalke et al. 2007a; Michalke et al. 2007b; Ellis et al. 2008). Gellein et al. (Gellein et al. 2007) developed a method to study the
protein binding patterns of trace elements in human CSF. Metalloproteins were
separated by SEC and measured off-line by ICP-SF-MS for Cd, Pb, Mn, Fe, Cu and Zn.
Michalke et al. (Michalke et al. 2007b) investigated the size distribution of Mn
transporters in CSF using SEC coupled with ICP-MS. Manganese was found to be
exclusively bound to low molecular weight species. In a subsequent study, Michalke et
al. (Michalke et al. 2007a) employed capillary zone electrophoresis coupled to ICP-MS
to monitor 13 Mn-binding species in CSF. Ellis et al. (Ellis et al. 2008) employed three
molecular weight cut-off ultra-filtration membranes (< 5 kDa, 5-50 kDa, > 50 kDa) to
separate human CSF components in samples from controls and from patients that had an
aneurismal subarachnoid hemorrhage. Only the > 50 kDa fraction was
chromatographically separated using AEC. All fractions were measured by ICP-MS for
Pb, Mg, Zn, Fe and Cu screening. Further characterization of fractions of interest was
conducted using nano-liquid chromatography-CHIP / ion trap mass spectrometry.

Considering that approximately one-third of proteins contain metal-binding sites
(Ghosh and Pecoraro 2005) and the majority of these accommodate Fe, Cu and Zn (the
three most abundant trace metals in the CNS) (Huidobro-Toro et al. 2008), surprisingly
little is known about their roles and mechanisms in neural activity (Takeda 2004).
Recent interest in the action of these metals and their speciation in the CNS may largely
be attributed to their involvement in neurodegenerative disorders as discussed in
Chapter 1.

Trace metals are typically present in the form of metalloproteins in neurons and
glial cells (Takeda 2004). However a small portion of trace metals reside in presynaptic
vesicles and are released along with neurotransmitters into the synaptic cleft (Assaf and
Chung 1984; Harttter and Barnea 1988; Kardos et al. 1989; Takeda 2004). Manganese is
involved in the metabolism of protein, lipid, and carbohydrate and serves as a cofactor
for enzymes such as glutamine synthetase, superoxide dismutase, decarboxylase, hydrolase and kinase (Takeda 2001; Takeda 2003). Magnesium metalloproteins are ubiquitous and are present in many metabolic cycles such as the citric acid cycle and glycolytic cycle (Cowan 2002). Copper and Fe metalloproteins are the principal source of oxidases, oxygenases and oxygen carriers in animal cells (Frieden 1976). This is of critical importance in the brain as it is particularly susceptible to oxidative stress due to its high energetic requirement and high levels of transition metals (Nalbandyan 1983; Mathie et al. 2006). The production of reactive oxygen species and consequent oxidative tissue damage has been regarded as playing a role in the etiology of neurodegenerative diseases such as AD, PD and ALS (Barnham et al. 2004).

As with Zn, there are very few quantitative data concerning the distribution of other metals such as Cu, Mn, Fe and Mg during neural plasticity. Huidobro-Toro et al. have raised the important issue of quantifying trace metals released from synaptic vesicles in order to gauge the magnitude of their roles (Huidobro-Toro et al. 2008). Furthermore, non-protein bound metal species have been largely attributed to the increasing evidence of trace metals functioning as allosteric modulators of ionotropic receptors (Assaf and Chung 1984; Hartter and Barnea 1988; Kardos et al. 1989; Takeda 2004). By combining ICP-SF-MS analysis with the separation techniques ultrafiltration and HPLC, the issue of quantification and the notion of unbound forms of these metals acting as neuro-modulators may be addressed.
5.2 Experimental and Analytical Procedure

5.2.1 Animals

The experiments were performed on 55 male out-bred Wistar rats aged 8-12 weeks. These animals were bred at The University of Newcastle Central Animal Facility and these studies were carried out with the approval of the Animal Care and Ethics Committee of The University of Newcastle.

5.2.2 Vibrissae removal

Vibrissae were plucked from the left side of the snout in a checker-board pattern whilst the animal was anesthetized using 1.5% isofluorane in oxygen. The rats were then left to recover for a period ranging from one hour to eight days. At the respective time point, tissue was extracted from the left and right barrel cortices and stored at -80°C in mannitol. A 24 h sham treatment group was also sampled in order to detect any non-specific effects related to the anesthesia and handling of the animals. These animals were exposed to the same conditions as the treatment groups with the exception of the vibrissectomy.

5.2.3 Statistics

The statistical significance of the ICP-SF-MS results was estimated by using one-way analysis of variance (ANOVA), with Tukey (HSD for unequal n) post-hoc comparisons test (Statistica 7, StatSoft Inc.).
5.3 Results

As reported for Zn fluctuation in chapter four, changes in the concentrations of the metals Cu, Mn, Fe and Mg induced by the unilateral checkerboard removal of snout vibrissae were also matched in the barrel cortex of both hemispheres for the controls, sham and all recovery time points (figure 5.1). Noteworthy was that both Cu and Mn recorded a significant decrease in concentration at 8 h from base levels in the contralateral cortex as recorded in the previous chapter for Zn.

The same procedures for establishing significant changes in metal concentrations were applied as used in Chapter four. Figure 5.1 presents the summary data for total Cu, Mn, Fe and Mn concentrations. With respect to total Cu, a statistically significant decrease in concentration was found at the 8 h recovery time in both contralateral and ipsilateral hemispheres. In addition, the contralateral hemisphere recorded a significant increase in Cu concentration at 96 h. Manganese recorded a significant decrease at 8 h in the contralateral hemisphere only.

Total Fe concentrations increased from a base level of 5 µg g$^{-1}$ in control and sham operated animals to around 12 µg g$^{-1}$ in the contralateral and ipsilateral cortex at the 24 h sampling point and this elevated Fe was maintained at the 96 h sampling (around 10 µg g$^{-1}$). Total Fe concentrations did not show a decrease at 8 hr after the vibrissectomy procedure as was the case for Zn, Cu and Mn. No significant changes in concentration were recorded for Mg. Low and high molecular weight determinations of Fe and Mg were not conducted as it is already established that the former is present in the brain in the high molecular weight form only (Piñero and Connor 2000; Wu et al. 2004) and the latter did not reveal any statistically significant change in total concentration across all recovery time points.
Figure 5.1 Total (a) copper, (b) manganese, (c) iron and (d) magnesium concentrations, respectively, in barrel cortex contralateral and ipsilateral to checkerboard vibrissectomy. The results represent mean values ± SD [Control (n = 8), 1 hour (n = 5), 8 hours (n = 4), 24 hours (n = 7), 24 hours sham (n = 5), 96 hours (n = 5)]. Significantly different time points from control are marked ($P < 0.05^*$, $0.01^{**}$).
Low molecular weight and protein bound Cu and Mn were distinguished by separation at 3 kDa and examined in controls and at 8 and 24 h after vibrissae manipulation (figures 5.2 & 5.3). A review of the data from both separated components revealed that the vibrissectomy-induced changes in total Cu concentration resulted from changes in the protein-bound fraction (high molecular weight component) only. Only the contralateral barrel field recorded a significant decrease in protein-bound Cu at 8 h. On the other hand, Mn showed significant decreases in both low molecular weight and protein-bound separation components. This decrease was observed in both contralateral and ipsilateral barrel fields.

Screening of Cu and Mn metalloproteins in controls and at 8 h in the high molecular weight component were carried out using AEC and the fractions collected (24 × 1 ml) were measured off-line for their Cu and Mn content (figures 5.4 & 5.5, respectively).
Figure 5.2 Low molecular weight (a) copper and (b) manganese concentrations (MWCO < 3 kDa) in barrel cortex contralateral and ipsilateral to checkerboard vibrissectomy. The results represent mean values ± SD [Control (n = 5), 8 hours (n = 5), 24 hours (n = 5)]. Significantly different time points from control are marked ($P < 0.05^*, 0.01^{**}$).
Figure 5.3 High molecular weight (a) copper and (b) manganese concentrations (MWCO > 3 kDa) in barrel cortex contralateral and ipsilateral to checkerboard vibrissectomy. The results represent mean values ± SD [Control (n = 5), 8 hours (n = 5), 24 hours (n = 5)]. Significantly different time points from control are marked ($P < 0.05^{*}$, $0.01^{**}$).
Figure 5.4 Distribution of protein-bound copper in the MWCO > 3kDa fraction (from control and 8 h post-vibrissectomy animals) separated by AEC and measured off-line using ICP-SF-MS. The results represent mean values ± SD [Control (n = 5), 8 hours (n = 5)].

While significant levels of Cu were measured in fractions 1-2, 8, 15 and 21-23, no known Cu metalloproteins were identified in them by subsequent measurement using ESI-TOF-MS. Only the Cu/Zn containing protein superoxide dismutase (SODC) was identified in fraction 10 in both control and 8 h animals. Aminopeptidase B (AMPB) was identified in fraction 13 of controls but is capable of binding Cu, Zn or Co. All known metalloproteins identified in the collected fractions and their identification parameters are shown in tables 5.1 and 5.2, respectively.

The chromatogram (Figure 5.4) shows a greater concentration of Cu metalloproteins in control animals compared to those in 8 hr post-vibrissectomy. This finding was also observed when measuring total copper content in the barrel cortex (Figure 5.1a) and in high-molecular weight separated fractions. At 8 h post-
vibrissectomy, Cu metalloproteins were absent (below detection limits) in fractions 1, 2, 8, 22, 23 and reduced in concentration in fraction 21 compared to controls.

The chromatogram of protein-bound Mn (Figure 5.5) was dominated by elevated levels of the metal in fractions 10 to 15 and to a lesser extent in fractions 20 to 21, although no known Mn proteins were identified in the latter. Fraction 3 recorded comparable levels of Mn between control and 8 h animals. Superoxide dismutase (SODM) was the only Mn metalloprotein recorded in fraction 3. NADP-dependent malic enzyme (MAOX) and isocitrate dehydrogenase (NADP) cytoplasmic (IDHC) were identified in fractions 10 and 11-12, respectively. Glutamine synthetase (GLNA) was identified in fractions 10-11.

Fraction 13 contained the Mn metalloproteins serine/threonine-protein phosphatase (PPP5), cytosol aminopeptidase (AMPL), and cytosolic non-specific dipeptidase (CNDP2) in control animals only. No Mn metalloproteins were identified in this fraction for 8 h post-vibrissectomy animals. Serine/threonine-protein phosphatase PP1-alpha catalytic subunit (PP1A) was identified in fractions 14-15 in both control and 8 h subjects. It should be pointed out that the peptide measurement of the alpha analog matched that of the beta (PP1B) and gamma (PP1G) analogs making them indistinguishable from each other. Xaa-Pro aminopeptidase 1 (XPP1) was identified in fraction 16 of 8 h post-vibrissectomy animals only.
Figure 5.5 Distribution of protein-bound manganese in the MWCO > 3kDa fraction (from control and 8 h post-vibrissectomy animals) separated by AEC and measured off-line using ICP-SF-MS. The results represent mean values ± SD [Control (n = 5), 8 hours (n = 5)]. Proteins identified by ESI-TOF-MS in the collected fractions are also displayed.
Table 5.1 Metalloproteins identified in 8 h post-vibrissectomy and control AEC separated fractions and the number of metal ions integrated into their protein structure.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>8 h post-vibrissectomy</th>
<th>Control</th>
<th>No. of metal ions</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>SODM</td>
<td>SODM</td>
<td>4 Mn</td>
</tr>
<tr>
<td>10</td>
<td>MAOX</td>
<td>MAOX</td>
<td>1-4 Mn and/or 1-4 Mg</td>
</tr>
<tr>
<td></td>
<td>SODC</td>
<td>SODC</td>
<td>2 Cu and 2 Zn</td>
</tr>
<tr>
<td>10-11</td>
<td>GLNA</td>
<td>GLNA</td>
<td>4-8 Mn</td>
</tr>
<tr>
<td>11-12</td>
<td>IDHC</td>
<td>IDHC</td>
<td>1-2 Mn and/or 1-2 Mg</td>
</tr>
<tr>
<td>13</td>
<td>PPP5</td>
<td>AMPL</td>
<td>2-4 Mn and 2-4 Fe</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CNDP2</td>
<td>1-6 Mn and 6-12 Zn</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4 Mn</td>
</tr>
<tr>
<td>14</td>
<td>AMPB</td>
<td></td>
<td>1 Cu or 1 Zn or 1 Co</td>
</tr>
<tr>
<td>14-15</td>
<td>PP1A, PP1B, PP1G</td>
<td>PP1A, PP1B, PP1G</td>
<td>2-4 Mn, 2-4 Fe</td>
</tr>
<tr>
<td>16</td>
<td>XPP1</td>
<td></td>
<td>4 Mn</td>
</tr>
</tbody>
</table>
Table 5.2 Identification parameters of metalloproteins by RPLC ESI-TOF-MS.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Tryptic peptide and adjacent residues</th>
<th>Sequence Position</th>
<th>Precursor m/z and Error (ppm)</th>
<th>Mascot Score</th>
<th>Mascot Expect</th>
</tr>
</thead>
<tbody>
<tr>
<td>SODM</td>
<td>K.GDVTTQVALQPALK.F</td>
<td>76-89</td>
<td>1439.8 (7)</td>
<td>82</td>
<td>$1.8 \times 10^{-1}$</td>
</tr>
<tr>
<td></td>
<td>R.DFGSFEK.F</td>
<td>124-130</td>
<td>828.4 (14)</td>
<td>37</td>
<td>$5.9 \times 10^{-3}$</td>
</tr>
<tr>
<td>MAOX</td>
<td>K.DLAFTLEER.Q</td>
<td>27-35</td>
<td>1092.6 (5)</td>
<td>55</td>
<td>$8.6 \times 10^{-3}$</td>
</tr>
<tr>
<td></td>
<td>R.LNSDFDR.Y</td>
<td>65-71</td>
<td>865.4 (35)</td>
<td>42</td>
<td>$1.7 \times 10^{-3}$</td>
</tr>
<tr>
<td></td>
<td>K.IVQDAYK.E</td>
<td>517-523</td>
<td>835.5 (15)</td>
<td>39</td>
<td>$3.4 \times 10^{-3}$</td>
</tr>
<tr>
<td></td>
<td>K.MATVYPEPQNK.E</td>
<td>526-536</td>
<td>1276.6 (18)</td>
<td>32</td>
<td>$2.1 \times 10^{-2}$</td>
</tr>
<tr>
<td>GLNA</td>
<td>R.ACLYAGIK.I</td>
<td>182-189</td>
<td>837.5 (12)</td>
<td>47</td>
<td>$4.5 \times 10^{-3}$</td>
</tr>
<tr>
<td></td>
<td>K.KGYFEDR.R</td>
<td>334-340</td>
<td>913.4 (17)</td>
<td>55</td>
<td>$8.3 \times 10^{-3}$</td>
</tr>
<tr>
<td></td>
<td>R.CIEEAIDK.L</td>
<td>269-276</td>
<td>919.5 (18)</td>
<td>31</td>
<td>$2.4 \times 10^{-2}$</td>
</tr>
<tr>
<td></td>
<td>K.LVFCEVFK.Y</td>
<td>96-103</td>
<td>983.5 (14)</td>
<td>35</td>
<td>$8.1 \times 10^{-3}$</td>
</tr>
<tr>
<td></td>
<td>R.DIVEAHYR.A</td>
<td>174-181</td>
<td>1001.5 (10)</td>
<td>42</td>
<td>$2.2 \times 10^{-3}$</td>
</tr>
<tr>
<td></td>
<td>R.MGDHLWVAR.F</td>
<td>214-222</td>
<td>1083.5 (19)</td>
<td>42</td>
<td>$1.6 \times 10^{-3}$</td>
</tr>
<tr>
<td></td>
<td>K.QMYMNLPQGEK.I</td>
<td>15-25</td>
<td>1353.6 (15)</td>
<td>54</td>
<td>$1.3 \times 10^{-3}$</td>
</tr>
<tr>
<td></td>
<td>R.LTGFHETSNINDFSAGVANR.S</td>
<td>300-319</td>
<td>2149.0 (12)</td>
<td>61</td>
<td>$1.9 \times 10^{-3}$</td>
</tr>
<tr>
<td>SODC</td>
<td>K.DGVANVSIEDR.V</td>
<td>93-103</td>
<td>1173.6 (11)</td>
<td>79</td>
<td>$3.5 \times 10^{-3}$</td>
</tr>
<tr>
<td>IDHC</td>
<td>R.ATDFVVPGPK.V</td>
<td>141-151</td>
<td>544.3 (15)</td>
<td>54</td>
<td>$1.3 \times 10^{-1}$</td>
</tr>
<tr>
<td></td>
<td>K.TVEAEAAAHGTVTR.H</td>
<td>302-314</td>
<td>447.9 (18)</td>
<td>31</td>
<td>$2.1 \times 10^{-2}$</td>
</tr>
<tr>
<td></td>
<td>R.SDYLNTFEMDKL</td>
<td>389-400</td>
<td>755.4 (20)</td>
<td>62</td>
<td>$1.8 \times 10^{-3}$</td>
</tr>
<tr>
<td>AMPB</td>
<td>K.KKPFWYTGQQAVALNR.A</td>
<td>162-176</td>
<td>1748.1 (97)</td>
<td>75</td>
<td>$8.1 \times 10^{-3}$</td>
</tr>
<tr>
<td>PPP5</td>
<td>K.VTITFMK.D</td>
<td>186-192</td>
<td>420.3 (80)</td>
<td>44</td>
<td>$7.1 \times 10^{-3}$</td>
</tr>
<tr>
<td></td>
<td>K.LLYPDHFHLLR.G</td>
<td>291-301</td>
<td>475.3 (96)</td>
<td>30</td>
<td>$2.3 \times 10^{-2}$</td>
</tr>
<tr>
<td></td>
<td>K.AFLEENQLDYIIR.S</td>
<td>413-425</td>
<td>812.5 (65)</td>
<td>79</td>
<td>$3.4 \times 10^{-1}$</td>
</tr>
<tr>
<td>AMPL</td>
<td>R.TLIEFLR.F</td>
<td>506-513</td>
<td>502.8 (61)</td>
<td>52</td>
<td>$1.7 \times 10^{-3}$</td>
</tr>
<tr>
<td></td>
<td>R.TFYGLHQDFPSVVVVGLGK.R</td>
<td>85-103</td>
<td>688.4 (76)</td>
<td>68</td>
<td>$3.9 \times 10^{-6}$</td>
</tr>
</tbody>
</table>
Table 5.2 continued

<table>
<thead>
<tr>
<th>Protein</th>
<th>Tryptic peptide and adjacent residues</th>
<th>Sequence Position</th>
<th>Precursor m/z and Error (ppm)</th>
<th>Mascot Score</th>
<th>Mascot Expect</th>
</tr>
</thead>
<tbody>
<tr>
<td>XPP1</td>
<td>K.GHIAVSAAVFPTGTK.G</td>
<td>450-464</td>
<td>486.0 (99)</td>
<td>30</td>
<td>$2.3 \times 10^{-2}$</td>
</tr>
<tr>
<td>CNDP2</td>
<td>R.LGGSVELVDIGK.Q</td>
<td>55-66</td>
<td>593.9 (75)</td>
<td>77</td>
<td>$4.6 \times 10^{-7}$</td>
</tr>
<tr>
<td>PP1A</td>
<td>R.AHQVVEDGYEFFAK.R</td>
<td>247-260</td>
<td>1638.9 (75)</td>
<td>67</td>
<td>$6.0 \times 10^{-6}$</td>
</tr>
<tr>
<td>PP1B</td>
<td>Protein matched the same peptide</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PP1G</td>
<td>Protein matched the same peptide</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

5.4 Discussion

Significant changes in Cu, Mn and Fe concentrations were found in the barrel-field representation of the snout vibrissae contralateral to a checkerboard vibrissectomy. No significant changes were observed in Mg concentrations at the recovery times compared to controls. Cu and Mn recorded a small increase in concentration one hour after the procedure followed by a significant decrease at 8 h compared to controls (a finding which was also recorded for Zn in the previous chapter). Copper and Mn levels increased above controls for the remaining recovery times (24 and 96 h). With the exception of Cu at 96 h, no other significant changes were observed for the two elements. Concentrations of Fe did not alter significantly until 24 h where a marked increase above controls was observed. This significant increase was maintained at 96 h. Concentration changes for Cu, Mn and Fe in the barrel cortex ipsilateral to the vibrissectomy showed the same pattern, and extent, as in the contralateral cortex for the respective elements. Discrepancies between the sum of cytosolic (low- and high-molecular weight fractions) and total metal content were noted.
and may be attributed to a combination of the normal variation between two independent experiments, two different groups of animals and sample preparation techniques (i.e. extraction and acidification versus total digestion).

The coincidental decrease in concentration of Cu with Mn and Zn at 8 h is a significant finding. All of these metals have been implicated as having modulatory roles in the CNS. It has been proposed that Cu may modulate neuronal excitability (Kardos et al. 1989; Collinge et al. 1994; Brown et al. 1997). Kardos et al. (Kardos et al. 1989) found that CuCl₂ decreased GABA-activated ³⁶Cl⁻ influx into synaptosomal membrane vesicles. Likewise, studies on frog dorsal root ganglion cells have shown that Cu ions depress GABA responses by blocking receptor activation and increasing the rate of receptor desensitization (Yakushiji et al. 1987). Moreover, Cu is thought to block GABAA receptors through the same mechanism as Zn (Narahashi et al. 1994; Trombley and Shephard 1996). Additionally, Cu (and Zn) has been found to be a strong inhibitor of NMDA receptors (Mayer et al. 1989; Doreulee et al. 1997; Trombley et al. 1998) and AMPA/kainite receptors are blocked by Cu (Weiser and Wienrich 1996). Manganese ions have been shown to be potent blocking agents of synaptic transmission at the neuromuscular junction in the frog (Meiri and Rahamimoff 1972b). Takeda et al. (Takeda et al. 2002) have revealed that Mn is co-released with glutamate into the synaptic cleft from glutamatergic neuron terminals and that it has an overall inhibitory action in synaptic neurotransmission (Takeda 2003). Manganese may also mimic the movement of Ca ions given the similarity of their ionic radii (Aschner and Aschner 1991) and is also known to block voltage-dependent Ca channels (Meiri and Rahamimoff 1972a), although the latter contradicts the former finding.

Like Zn, vesicular ionic Cu and Mn are believed to be released from neurons following membrane polarization via exocytosis in a Ca^{2+}-dependent manner (Assaf and
Chung 1984; Hartter and Barnea 1988; Kardos et al. 1989; Takeda et al. 2002; Takeda 2003). This study, however, could only attribute the significant decrease in Cu concentration to protein-bound forms. Furthermore, the decrease was statistically significant in the barrel cortex contralateral to the vibrissectomy only. Conversely, protein-bound and unbound Mn recorded a significant decrease at 8 h post-vibrissectomy in both barrel cortex contralateral and ipsilateral to the vibrissectomy.

Again, like Zn, unbound Cu is more likely to be present in a physiological environment chelated to small molecules such as cysteine or histidine with the majority of the metal present in protein-bound form (Neumann and Sass-Kortsak 1967). On the other hand, approximately 60% of Mn is reported to be unbound in the plasma (May et al. 1997) and that the metal is readily transported into the brain as the free ion (Rabin et al. 1993).

Relatively little is known regarding the transport of Cu throughout the CNS; however, there is evidence that the Cu-transporting ATPases ATP7A and ATP7B have a fundamental role in this metal’s distribution (Schlief et al. 2005; Mathie et al. 2006; LaFontaine and Mercer 2007). These proteins are mobilized from the late Golgi compartment triggered by increases in Cu confined within cytoplasmic vesicular compartments for subsequent excretion (Mathie et al. 2006; Lutsenko et al. 2007). ATP7A and ATP7B may also catalyse the transfer of Cu to these intracellular compartments as well as incorporate Cu directly into the structure of Cu-dependent enzymes (e.g. SODC) (Harris 2000). This mechanism allows the maintenance of intracellular Cu homeostasis. Notwithstanding the above, the only Cu metalloproteins identified were SODC and AMPB which showed no difference in concentration between control and 8 h post-vibrissectomy. Given the pI of ATP7A and ATP7B, at approximately 6.4 compared to SODC and AMPB at < 6, it is expected that the ATPase
proteins would elute off the AEC column in subsequent fractions (i.e. fraction 15, or fractions 20-23). Only fractions 1-2, 8 and 20-23 showed decreased Cu levels in 8 h post-vibrissectomy animals compared to those in controls and, if any Cu metalloproteins were successfully identified in them, would likely reveal the metalloproteins linked to the significant depression of Cu at 8 h.

Several Mn metalloproteins were identified, with MAOX, GLNA, IDHC and PP1 all showing reduced levels in 8 h post-vibrissectomy animals compared to controls based on recorded Mn concentrations in the respective fractions. The Mn metalloproteins PPP5, AMPL and CNDP2 were identified in controls only. MAOX catalyzes the reduction of NADP to NADPH (Sauer 1973) with NADPH being primarily involved in protection against reactive oxygen species. IDHC catalyzes oxidative decarboxylation of isocitrate to 2-oxoglutarate (Murakami et al. 1997), a reaction which is a part of the citric acid cycle. GLNA catalyzes the conversion of glutamate to glutamine and accounts for approximately 80% of total Mn in the brain (Takeda 2003). Glutamate is considered to be the main excitatory neurotransmitter (Huidobro-Toro et al. 2008) and is responsible for activating NMDA and AMPA/kainite receptors.

The serine/threonine-protein phosphatases, PP1 and PPP5, are only expressed in the brain and take part in a range of signalling pathways that modulate neural activity (Price and Mumby 1999; Janssens and Goris 2001; Winder and Sweatt 2001). Winder and Sweatt (Winder and Sweatt 2001) have outlined three means by which phosphatases can participate in synaptic plasticity. Firstly, regulation of neuronal excitability or NMDA receptor activation, secondly, participation in the initial triggering mechanisms for synaptic plasticity subsequent to NMDA receptor activation, and lastly, participation in the persistent maintenance of the depressed or potentiated
state. Additionally, Winder and Sweatt (Winder and Sweatt 2001) have indicated that LTP induction is associated with the inhibition of PP1. Earlier work supports this link between LTP and activity-dependent suppression of PP1 activity (Blitzer et al. 1995; Thomas et al. 1996). Conversely, the induction of LTD is associated with a transient increase in PP1 activity (Thiels et al. 2000). It has been suggested that PPP5 modulates glucocorticoid receptor signalling (Chen et al. 1996) and may terminate physiological responses to oxidative stress (Morita et al. 2001).

Aminopeptidases (including AMPL) are a class of exopeptidases that catalyze the removal of single NH$_2$-terminal amino acid residues from peptide substrates (Wilk et al. 1998; Gil et al. 2001). While membrane-bound aminopeptidases are well-established, little is known regarding the role of soluble aminopeptidases, such as AMPL (Mantle 1992). CNDP2 catalyzes the hydrolysis of a variety of dipeptides including L-carnosine (which is known to scavenge reactive oxygen species) (Otani et al. 2008).

The overall reduction in concentration of Mn metalloproteins is either a concerted physiological response or simply a result of the reduced availability of Mn ions and low-molecular weight Mn species for incorporation into proteins. The former view is not able to be verified with the current data as there is an assumption that the fluctuation of Mn in a given fraction is solely attributed to the coincidental identification of a Mn containing protein. Essentially, the high sensitivity of ICP-SF-MS for metals is limited by the variable sensitivity of the different peptides measured using ESI-TOF-MS.

The simultaneous depression of Cu, Mn (and Zn) at 8 h post-vibrissectomy may produce a net excitatory effect given that these metals have a net inhibitory effect on neural activity at relatively higher concentrations. If the decrease in Mn concentration is attributed to reduced PP1 levels then this may signal the induction of LTP. Wright (Wright 1984) examined the effect of Zn and other cations on the activity of neurons in
the rat frontoparietal cortex. Interestingly, when Mn was applied simultaneously with Zn, its depressant effect on cortical neurons was not blocked by Zn. Given the findings above, Mn and Zn do not appear to impede one another’s action.

5.5 Conclusion

Copper, Mn, Fe and Mg fluctuation were investigated in the barrel cortex of rats contralateral and ipsilateral to partial vibrissae removal (checkerboard pattern plucking) with subsequent analysis using ICP-SF-MS and ESI-TOF-MS for metal quantification and protein identification, respectively. This allowed measurement of total metal content and separated low molecular weight (free) and protein bound components along with the protein identification. A significant decrease of Mn and Cu at 8 h after whisker plucking was revealed in both deprived and non-deprived barrel cortices. Changes to Cu are attributable to protein-bound species and changes to Mn may be attributed to both protein-bound and non-protein bound species. The significant decrease of the metals Cu and Mn (along with Zn) at 8 h post-vibrissectomy may indicate a synchronised compensatory mechanism of the CNS for reduced sensory input given their role in modulation of neural activity.
Chapter Six: Conclusion and Future Directions

6.1 Conclusion

Three aims were stated in the introduction of this thesis. It is worthwhile considering the extent to which each of these aims was met:

1) To develop and validate generic ICP-SF-MS methods for the sampling, sample preparation and multi-elemental analysis of brain tissue of rats in order to benchmark/establish their normal or healthy trace metal concentration levels and distribution.

A reliable standardized method for the analysis of rat brain tissue using ICP-SF-MS was developed based on already well-established guidelines for ICP-AES and ICP-Q-MS sample preparation and operation. Development of a measuring paradigm using ICP-SF-MS was found to be considerably easier than for these alternative techniques given the absence of interfering species when using its higher resolving power. The use of microwave digestion ensured a complete digestion through its ability to digest at high pressures and; at temperatures well above the boiling point of the acid.

More importantly than the sample preparation and instrument operation is the sampling procedure. As discussed in chapter three, much of the variation with the major elements (i.e. Cu, Fe, Zn) between different investigators may be attributed to the sampling methodology; specifically the perfusion technique. By performing intracardial perfusion, blood was thoroughly washed out; doing so at physiologically-matched low pressures reduced the risk of rupturing blood vessels. Equally, control of external contamination was critical in order to obtain reproducible results, particularly when
working with relatively small amounts of tissue. This was addressed in this work through the use of a laminar flow hood and plastic dissection tools. The choice of well-defined brain regions combined with the above measures ensured a robust and reliable method for performing investigations on the CNS and also to allow meaningful comparisons and conclusions to be made.

Tissue concentrations of Ag, Cd, Hg, Pb, Bi, U, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As and Se in the olfactory bulb, telencephalon, pons and medulla, superior/inferior colliculus, diencephalon, hippocampus and cerebellum brain regions were measured. The major elements Fe and Cu were uniformly distributed across the eight brain regions investigated. Likewise, Mn was uniformly distributed across the eight brain regions. Conversely, Zn was shown to be significantly more concentrated in the cerebellum and in the telencephalon than the other brain regions. Cobalt was detected in only six brain regions at very low concentrations showing little association with any of the brain regions. Similarly, V and Cr were present at low concentrations across the four and three brain regions, respectively.

Nickel was strongly associated with the pons and medulla and hippocampus, and Se had the strongest association with the inferior colliculus and to a lesser extent with superior colliculus and hippocampus. The non-essential trace elements (As, Ag, Hg, Pb, U) were more concentrated in the inferior colliculus with Pb being the most characteristic element in this region. Mercury was also highly concentrated in the inferior colliculus, but also with pons and medulla. It was suggested that the higher concentration of Se in the inferior colliculus, where a greater array of non-essential elements reside, may be of toxicological significance considering its detoxifying and antioxidant properties.
2) To investigate trace metal fluctuation (in particular Zn, Cu, Mn, Fe and Mg) in rat brain tissue after reduced sensory input by vibrissae removal using ICP-SF-MS.

With a standardized ICP-SF-MS method in place for CNS investigations, trace metal fluctuation in the rat cerebral cortex after vibrissectomy could be reliably quantified. The high sensitivity of ICP-SF-MS allowed as little as 30 mg of tissue was required to be analyzed as well as provide increased study of functionality by measuring trace metal fluctuation in the barrel cortex only. The metals Zn, Cu, Mn and Fe were shown to fluctuate from control concentration levels after reduced sensory input. Magnesium concentrations did not alter significantly from control. The fluctuations of less concentrated elements were unable to be reliably measured as their concentration levels were relatively close to the detection limits of the instrument. The temporal coincidence of the depression of the metals Zn, Cu and Mn is significant as this has not been previously recorded in the literature – each of these metals have been implicated as potent neuromodulators and as important elements in the progression of neurodegenerative diseases.

3) To develop methods of screening for metalloproteins using a combination of LC-MS instrumentation (i.e. HPLC/ICP-SF-MS and HPLC/ESI-TOF-MS) and subsequently investigate protein-bound and unbound metal species in rat brain tissue after reduced sensory input by vibrissae removal.

Expanding on the outcome of the second aim, the different forms of the metal contributing to the fluctuation after reduced sensory input were distinguished. Key to
the success of this component of work was the combination of mass spectrometry instrumentation combined with HPLC.

ICP-SF-MS is a sensitive elemental detector for screening a given element in biomolecules. By quantifying increases or decreases in protein-bound metals, ICP-SF-MS effectively vets any insignificant metalloproteins from those of interest thereby reducing the effort for further studies using organic MS techniques to identify a protein-bound metal.

Initially, the use of ultrafiltration allowed the protein-bound forms of Zn, Cu and Mn and their low-molecular weight forms to be distinguished from one another. This served as the first stage of the metalloprotein screening process. Any significant quantitative differences between control metal concentration levels and those in treatment samples in the > 3 kDA molecular weight cut-off fraction determined whether protein mass spectrometry was necessary. Fluctuation of Zn in the barrel cortex was attributed to low-molecular weight bound species, a finding which helps to interpret early studies of rodent whisker-manipulation outcomes utilizing Zn histochemical staining. Conversely, significant Cu fluctuation could be attributed to protein-bound species only. Manganese recorded significant changes in both protein-bound species and low-molecular weight bound species.

Only two Cu metalloproteins were successfully identified, however, they could not be attributed to the depressed level in concentration at the 8 h recovery time. Several Mn metalloproteins were identified and implicated in the depression of Mn concentration levels at the 8 h recovery time. While the fluctuation of Mn in given HPLC fraction could not be solely attributed to a coincidental identification of a Mn metalloproteins, the findings of this methodology serves to identify metalloproteins of interest for future investigations.
6.2 Future Directions of Methodology

ICP-MS offers great possibilities for synergistic studies and identification of inorganic tracers in biomedical research. The challenge of future research is to interleave knowledge about the biochemistry of metal ions in order to unravel how complex living systems function. In particular, an understanding of metal neurochemistry will focus on the role metal ions play in synaptic transmission, in memory formation, and in the causes and treatments of neurological diseases.

This work presents a start to measuring metal fluctuation, with a known model of neuronal plasticity, using ICP-SF-MS. This work demonstrates a bioanalytical tool and methodology, which is capable of mapping metal fluctuation as a function of time, thereby enabling researchers to search at appropriate time-points (after a perturbation) for proteins, which control/allow growth. Thus, this general methodology for future metal neurobiological investigations can involve: (i) use of known models of neuronal plasticity; (ii) employment of the ICP-SF-MS to map the time courses of metal fluxes; (iii) use HPLC/ICP-SF-MS, ESI-MS and MALDI-TOF to identify the metal bound proteins, which control/allow growth.

In the future, quantum leaps in advances of trace metal detection in metallonurochemistry will be driven largely by instrument design. Ion-electron coincidence experiments have been commonplace for decades. The push is on to develop metal-metal ligand coincidence experiments; that is, to extract the metal ion from a ligand (such as a protein) and so determine its oxidation state passively, as well as determine unambiguously the metal ligand complex. This coincidence instrument would ideally have high sensitivity and selectivity. Undoubtedly only an interdisciplinary team could utilize it to its fullest capability.
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