INVESTIGATION OF THE NEURONAL MARKER N-ACETYLASPARTATE AS A POTENTIAL BIOMARKER FOR NEUROLOGICAL DISEASES

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Abstract

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Investigation of the neuronal marker A-acetylaspartate as a potential biomarker for neurological diseases
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N-acetylaspartate (NAA) is known to be present in high concentrations throughout the whole CNS. It is believed to have four basic functions in the brain; an organic osmolyte, a source of acetate for myelin synthesis, an energy source and a precursor for N-acetylaspartate-glutamate (NAAG). Because of NAA's ability to be measured both *in vivo* and *ex vivo* as well as its high concentration it is believed that it has potential to be used as a biomarker for neuronal loss/dysfunction.

The present studies were utilised to develop the potential of NAA as a biomarker using a variety of techniques and neurological diseases both in pre-clinical and human post-mortem studies.

Using phencyclidine (PCP) to induce schizophrenia-like symptoms we showed that reductions in the levels of NAA in the frontal cortex and ventral hippocampus are observed in a post-mortem analysis of rat brain tissue. This reduction is not ameliorated following chronic administration of the atypical antipsychotic olanzapine. A deficit in cell density in the frontal cortex was also found following cresyl violet staining. Again this deficit was not ameliorated following chronic administration of atypical antipsychotic olanzapine.

With *in vivo* analysis using Magnetic Resonance Spectroscopy (MRS) we replicated these findings 6 weeks post PCP treatment. This neuronal dysfunction was supported with behavioural data showing a deficit in the PCP group in the novel object recognition (NOR) task. Following behavioural testing a post-mortem analysis was undertaken on the cohort mirroring the reduction in NAA using the HPLC method of analysis in the frontal cortex.

Following High Pressure Liquid Chromatography (HPLC) analysis of human brain samples provided by the Stanley Foundation we found a reduction in the level of NAA in human schizophrenic brain tissue when compared to controls. These deficits were found in the frontal cortex and further support the findings in the preclinical experiments.

In the final studies we focused on a preclinical rat model of relevance to Alzheimer's disease. Using acute administration of soluble Amyloid Beta$(A\beta)_{1\text{-}42}$ oligomers we demonstrated a model of early stage Alzheimer's. We observed a cognitive deficit in the NOR task however no reduction in NAA in the brain regions examined. These finding are indicative of a synaptic dysfunction however no neuronal dysfunction at the early stage of the disease.

Taken together, these findings suggest that NAA shows promise as a biomarker for neuronal dysfunction in neurological diseases that can be used in pre-clinical and clinical setting with both *in vivo* and *ex vivo* applications. We also demonstrate the potential translational use of NAA as a biomarker for assessing treatment efficacy in relation to the underlying pathological changes in the brain.

Declaration

I (Samuel Marsh) declare that no portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification at this or any other university or other institute of learning.

Copyright Statement

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Abbreviation List

1H NMR - Proton Nuclear Magnetic Resonance

5-HT - Serotonin

ACC - Anterior Cingulate Cortex

AD - Alzheimer's Disease

ADC - Axonal Diffusion Coefficient

ADCS-ADL - Alzheimer's Disease Cooperative Study of Daily Living

ADDL's-Aβ - Amyloid Beta Derived Diffusible Ligands

AMPA - α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

ANOVA - Analysis of Variance

APES - 3-aminopropyltriethoxysilane

APP - Amyloid Precursor Protein

ASPA - Aspartoacylase

ATP - Adenosine Triphosphate

Av - Absolute Values

Aβ - Amyloid Beta Protein

BACE 1 - β Secretase APP cleaving enzyme 1

BDNF - Brain Derived Neurotrophic Factor

BSA - Bovine Serum Albumin

cAMP - Cyclic adenosine monophosphate

CANTAB - Cambridge Neuropsychological Test Automated Battery

C*f* - Correction Factor

cGMP - Cyclic guanosine monophosphate

Cho - Choline

CNS - Central Nervous System

Cr - Creatine

CSF - Cerebral Spinal Fluid

CT - Computerised Tomography

DA - Dopamine

DI - Discrimination Index

DLPFC - Dorsolateral Prefrontal Cortex

DSM-IV - Diagnostic and Statistical Manual (of mental disorders) 4

DSST - Digital Symbol Substitution Test

DTI - Diffusion Tensor Imaging

FC - Frontal Cortex

GABA - γ-aminobutyric acid

GM - Grey Matter

HA - Hunters Angle

HPLC - High Pressure (Performance) Liquid Chromatography

Hz - Hertz

i.p. - intraperitoneal route of administration

ICV - Intracerebroventricular

IQ - Intelligence Quotient

ITI - Inter Trial Interval

LSD - Least Significant Difference

LTD - Long Term Depression

LTP - Long Term Potentiation

LX - Line Crossing

M - Muscarinic

MATRICS - Measurement And Treatment Research to Improve Cognition In Schizophrenia

MCI - Mild Cognitive Impairment

MFC - Medial Frontal Cortex

MK-801 - Dizocilpine

MRS - Magnetic Resonance Spectroscopy

MS - Multiple Sclerosis

NAA - N-acetylaspartate (N-acetyl-L-Aspartic Acid)

NAAG - N-acetylaspartylglutamate

nACH - nicotinic Acetylcholine

NFT - Neurofibrillary Tangles

NMDA - N-methyl-D-aspartate

NOR - Novel Object Recognition

PCP - Phencyclidine

PET - Positive Emission Tomography

PFC - Prefrontal Cortex

PMI - Post Mortem Interval

PSEN 2 - Presenilin 2 Gene

PSEN1 - Presenilin 1 Gene

Q_{NAA} - Quantity of NAA

Sc - Sample Content

SEM - Standard Error of the Mean

SP - Senile Plaques

STEAM - Short Echo Stimulated Echo Acquisition Mode

T - Tesla

TBI - Traumatic Brain Injury

T_F - Time spent on the familiar object

T_N - Time spent on the novel object

UV - Ultraviolet

VOI - Volumes of Interest

WCST - Wisconsin Card Sorting Test

WHO - World Health Organisation

WM - White Matter

βCTF - β C-terminal Fragment

Chapter 1

General Introduction

1.1 N-Acetylaspartate (NAA)

The first reports of N-acetylaspartate (NAA) that have been noted is the work of Tallan using the brains of cats and rats (Tallan et al., 1956). NAA is still something of a mystery as there is virtually no consensus on its principle metabolic or neurochemical functions. There is however a general consensus on one point; NAA is not thought to be a neurotransmitter or neuromodulator and that it is released synaptically upon neuronal depolarisation. It is present in high concentrations throughout the entire CNS and is the second most abundant amino acid after glutamate, making up 1.0% of the dry weight of the brain (Arnold et al., 2001; Baslow, 2000; Birken and Oldendorf, 1989), with inter-neuronal concentrations being between 10 and 20 mM. NAA has also been seen to have a very high half life in the human brain of only 16.7 hours (Baslow, 2002).

1.2 Structure, Metabolism & Function of NAA

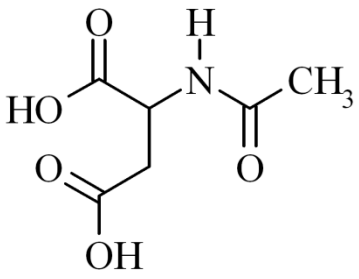

Figure 1: Molecule of NAA

It is widely known that NAA is synthesized in the neuronal mitochondria (Patel and Clark, 1979), (Bates et al., 1996). Other evidence that has been presented which

supports the neuronal origin of NAA can be seen from studies with tumours of glial origin that contain no NAA and those of neuronal origin that do (Nadler and Cooper, 1972), also from the effect of neuro toxins that have been shown to deplete NAA levels (Koller et al., 1984) as well as immunohistochemical studies, work which demonstrated that NAA was localised to neurones in the rat CNS (Moffett et al., 1991), (Simmons et al., 1991). Taken together these studies suggest that NAA is a major chemical constituent of the CNS and also a marker of healthy neurones in the adult brain.

NAA is synthesised in the brain through the acetylization by acetyl coenzyme A of free aspartate by the enzyme L-aspartate NAA synthase (Goldstein, 1959) and is catabolised by the enzyme aspartoacylase. Cellular localization studies of NAA indicate that the NAA catabolic enzyme was myelin-associated, suggesting that it is found primarily in oligodendrocytes (Kaul et al., 1991). Figures 2 and 3 show the molecular formation process of NAA and then the location and pathways in a neuron relating to the aforementioned work on NAA.

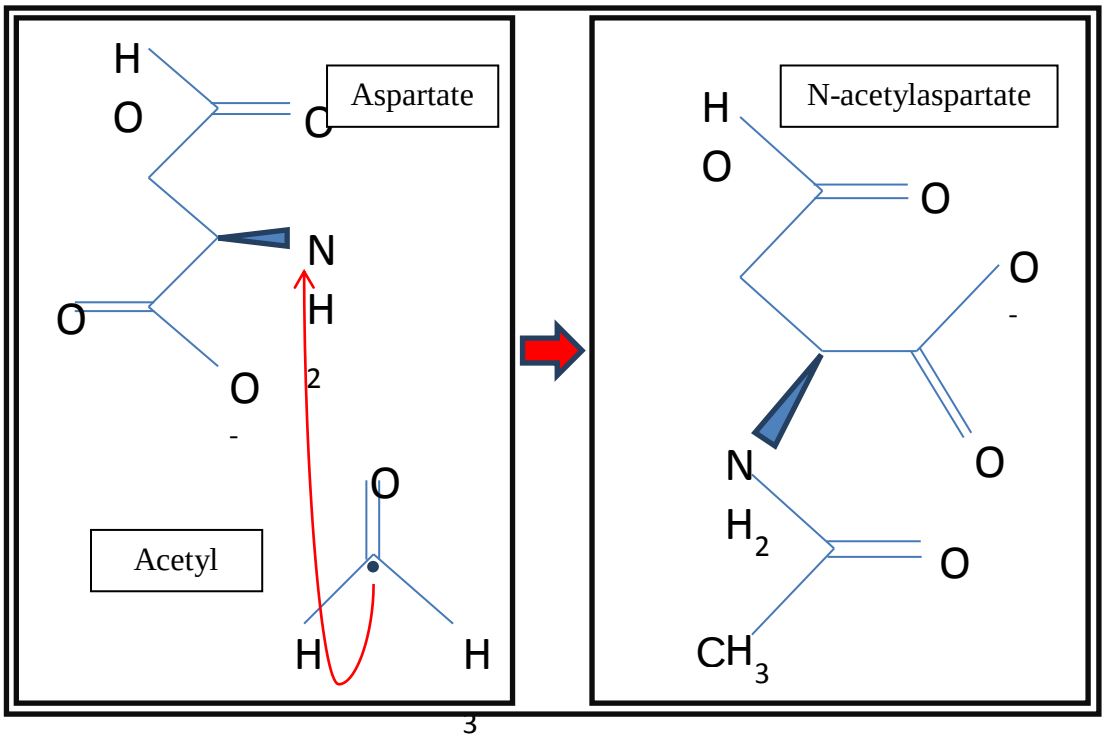

Figure 2: Schematic description of the structures and acetylization of aspartate into NAA in the mitochondria by NAA synthase.

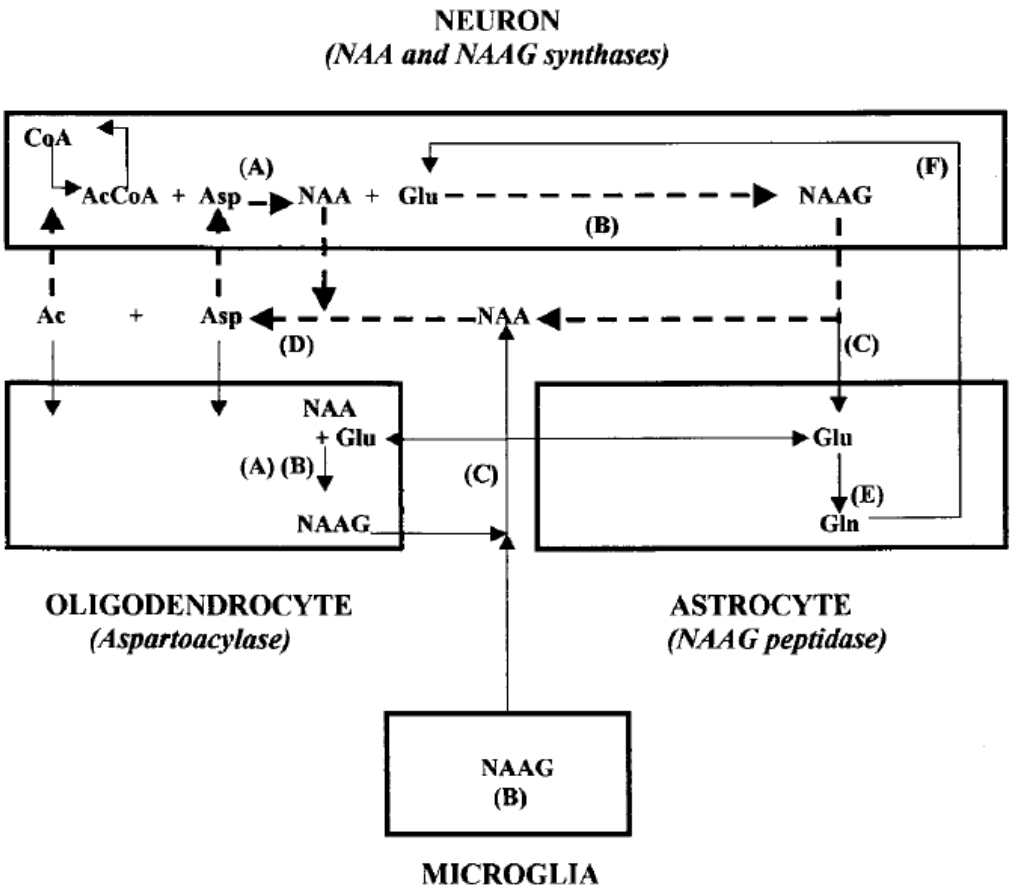

Figure 3: NAA & NAAG Synthesis

Taken from (Baslow, 2000). Tricellular brain metabolic NAA-NAAG cycle operating between neurons, astrocytes and oligodendrocytes. Enzymes involved in metabolic sequences are (A) NAA synthase, (B) NAAG synthase, (C) NAG peptidase, (D) aspartocyclase, (E) Gln synthase and (F) glutaminase. AC, acetate; AcCoA, acetyl-coenzyme A; CoA, Coenzyme A.

NAA is catalyzed by the enzyme aspartoacylase (ASPA) by deacetylation. The enzyme was first identified in supernatant fractions from hog kidney homogenates with specificity for NAA with little activity towards other acetylated amino acids (Birnbaum et al., 1952). ASPA was shown to be expressed in higher levels in white matter than in grey matter (Kaul et al., 1991). *In vitro* work in cell culture shows that the ASPA enzymatic activity is present in oligodendrocytes but not neurons or astrocytes (Baslow et al., 1999). It is the miscoding of the ASPA gene, with an A to C substitution at nucleotide 854 this causes a reduction of ASPA activity to 2.5% of

normal levels. There are also two less common mutations (at nucleotide 693 and 914) that result in a complete loss of ASPA activity (Kaul et al., 1994) and are linked to Canavan's Disease (See 1.4.1).

There have been four basic hypothesis for the principle role of NAA in the nervous system (Moffett and Namboodiri, 2006b), (Chakraborty et al., 2001), (Baslow, 2003):

1. An organic osmolyte that counters the "anion deficit" in neurones, or a co-transport substrate for a proposed "molecular water pump" that removes metabolic water from neurones.

This was first noted (Tallan, 1957) by showing that NAA accounted for more than 10% of the total anion content of brain tissue in birds and mammals. However, for the molecular water pump theory to be verified a protein, or protein complex, that co-transports NAA and water from neurons needs to be identified and characterised.

2. A source of acetate for myelin lipid synthesis in oligodendrocytes.

It was in 1987 that (Hagenfeldt et al., 1987) proposed that the dysmyelination in Canavan's disease was due to the failure of NAA to carry acetyl groups from the mitochondria to the cytosol for lipogenesis. Work done by (Mehta and Namboodiri, 1995) showed that radiolabeled NAA and acetate were incorporated into acetyl CoA and brain lipids. To support this (Chakraborty et al., 2001) showed that NAA

injected into the axon of optic nerves is incorporated into the ensheathing myelin. Taken together, these studies support a role for NAA in myelin synthesis.

3. An energy source in neurones.

The first evidence that linked NAA to energy metabolism was by (Patel and Clark, 1979). Their finding was that brain derived mitochondrial preparations were distinct from those derived from other tissues in that they synthesized large amounts of NAA. There seemed to be a reciprocal relationship between aspartate efflux and NAA efflux when rat brain mitochondria oxidised glutamate and malate in the presence of various concentrations of pyruvate. With a pyruvate deficit there was no source of acetyl CoA and therefore no NAA efflux was detected. Increasing pyruvate concentrations caused a decrease in aspartate efflux and an increase in NAA efflux. (Clark, 1998) showed a biochemical coupling between NAA synthesis and energy production in brain mitochondria. This coupling relationship has been subsequently substantiated by additional reports noting decreases in NAA in a number of conditions of impaired energy metabolism in the brain.

4. A precursor for the biosynthesis of N-Acetylaspartylglutamate (NAAG).

NAA has been suggested to be an intermediate metabolite in the formation of NAA-glutamate (NAAG) (Baslow, 2000), a known functional neurotransmitter (Neale et al., 2011). While NAA may be an intermediate in the formation of NAAG, the relatively large concentration of NAA in the brain seems superfluous for the relatively small concentration and flux of NAAG. NAAG is about 10% of the

concentration of NAA (Passani et al., 1997) and NAA's role as a neurotransmitter is unclear (Coyle, 1997; Urenjak et al., 1992; Yan et al., 2003).

Other possible roles also exist for NAA other than the four listed above. In one study NAA has been suggested to form a complex with tRNA involved in protein synthesis (Clarke et al., 1975). NAA has also been found to be linked to an increase in cAMP and cGMP levels in minced cortical preparations (Burgal et al., 1982). In a more recent study NAA was suggested to be an endogenous ligand for G protein coupled metabotrophic glutamate receptors, where NAA (in a dose-dependent manner) was shown to induce neuronal depolarisation in dissociated hippocampal neurons (Yan et al., 2003). NAA has also been seen to be present and released by mast cells implicating a possible immune function (Burlina et al., 1997).

Suggestions have also been made that NAA could have some form of anti-inflammatory role (Rael et al., 2004). As will be discussed in future chapters, it is widely accepted that low levels of NAA, e.g. as detected my MRS, have been interpreted to indicate neuronal/axonal loss, or compromised neuronal metabolism.

With all of the theories above, no one or group of main roles of NAA can be determined. One thing that can be determined is that NAA has a vital role in neuronal functioning and it is this that will allow us to investigate its use as a translational biomarker.

The main support, to the use of NAA as a biomarker. comes from work with Canavan's disease. High levels of NAA were found in the brains of many Canavan's disease patients suggesting that excess NAA levels produce detrimental effects in the CNS (Moffett et al., 2007). In other disorders it was found that levels of NAA, in various brain locations, associate with neuronal health or integrity in that region. As such decreased levels of NAA, as detected by MRS, have been interpreted to indicate neuronal / axonal loss, or compromised neuronal metabolism.

It is the focus of this thesis to investigate the potential use of NAA as a translational biomarker to analyse changes in neuronal function in brain regions in different disease states. It will also address the ability of NAA as a potential biomarker for treatment efficacy in terms of improving the underlying pathology alongside reversal of behavioural measures in preclinical models.

1.3 Methods of Analysis of NAA

At present there are three distinct techniques for measuring NAA. There are those that use the non-invasive (clinical) applications of Magnetic Resonance Spectroscopy in the brain. Then there are those that employ research into the neurochemistry of NAA (Invasive - post-mortem analysis, HPLC and immunohistochemistry).

1.3.1 Magnetic Resonance Spectroscopy (MRS)

MRS proves to be an increasingly useful measurement technique due to its clinical application and its non-invasive methodologies. The power of MRS detection technique is that NAA shows itself to be the strongest signal on an MRS trace with high concentrations in neurons (Clark, 1998; Urenjak et al., 1992). Further to this the correlation of NAA concentrations with neuronal damage (Lentz et al., 2005) shows increasing promise of use as a diagnostic method. There is a large amount of current literature that uses MRS to show changes in the brain of patients with a number of psychiatric conditions, including; schizophrenia, dementia, obsessive compulsive disorder, to name a small selection. NAA reductions have been mostly seen in locations of grey matter reductions (Paslakis et al., 2014). MRS is covered in greater detail in chapter 3 of this thesis.

1.3.2 HPLC Analysis of NAA

Whereas MRS provides a non-invasive approach to the measure of NAA, there are limitations on the methodology (see Chapter 3, Introduction). When an invasive approach can be used (post-mortem animal models or human cadaver tissue) then the HPLC method can be a useful tool. The first use of HPLC, as an analysis of NAA, can be seen in the work by (Koller et al., 1984) to determine NAA levels after brain lesions. The most important benefit of using HPLC over MRS is that it provides an absolute value for the level of NAA in a specific region, rather than in MRS using creatine or cerebral water as an internal reference (Bertolino et al., 1998), which provides a more relative method of analysis. HPLC has been used to calibrate the MRS method to determine the accuracy of an *in vivo* estimation of absolute NAA

concentrations by magnetic resonance spectroscopy (MRS) (Fatouros et al., 2000) due to its absolute values. Even though it produces absolute values it is also the fact that complicated sample preparations, using ion exchange columns, must be used before the HPLC assay (Koller et al., 1984; Sager et al., 1995). This process can be time consuming and requires a large amount of calibration before experiments can be run. Work that has been carried out by (Harte et al., 2005; Harte et al., 2004) show a similar method that will be used later in this thesis to determine NAA levels.

1.3.3 Immunohistochemical Analysis of NAA

Antibodies specific to protein-coupled NAA were not discovered until 1991. Monoclonal antibodies were discovered by (Simmons et al., 1991) and polyclonal by (Moffett and Namboodiri, 2006a; Moffett and Namboodiri, 1995; Moffett et al., 1991; Moffett et al., 1993). Work done showed that NAA is present in neurons and their processes and that the levels vary substantially between different neuronal populations with a great range of varying intensities between different neuronal types. For example in the cerebral cortex and the hippocampus NAA immunoreactivity was seen to be present in most pyramidal neurons (Moffett et al., 1993). Based on immunohistochemical findings NAA is present in most neuronal cell populations. It is, however, found that the intracellular concentrations appears to vary greatly between neuronal groups (Moffett and Namboodiri, 1995). Immunohistochemical studies confirmed early reports that NAA concentrations were substantially higher in cerebral grey matter than in white matter. Further, they confirmed that NAA did not exhibit a significant concentration gradient along the rostro-caudal axis of the CNS. No immunocytochemical electron microscopic

studies have been carried out on the sub cellular localization of NAA, but light microscopic studies suggest that NAA is found diffusely throughout neuronal cytoplasm, and is also exceptionally concentrated in small punctate structures within neuronal cell bodies and basal dendrites, that may represent clustered neuronal mitochondria (Moffett and Namboodiri, 2006a; Moffett and Namboodiri, 1995). NAA has been shown to be present at varying concentrations in different nervous tissues, with higher levels of NAA in the forebrain and lower levels in the hind brain and spinal cord of adult animals (Miyake et al., 1981; Tallan, 1957).

1.4 Diseases in relation to NAA

NAA has been shown previously to be linked to diseases and disease states in brain tissue. Canavan's disease is directly linked to NAA as is explained below. However, it is the only disease directly linked to NAA concentration in the brain. Other diseases only show a variation in NAA concentration due to other factors depending on the individual neurological condition.

1.4.1 NAA in Canavan's Disease

Canavan's disease is important when looking at NAA as it is directly linked to the molecule and, as such, gives us an insight into some of its potential function in the brain. Canavan's disease is known as a rare leukodystrophy, or as a spongy degenerative disease involving white matter. It was first observed in an infant and noted by Myrtelle May Moore Canavan in 1931. Infants with Canavan's disease

seem normal at birth however display delayed development and hypertonia. At two to six months of age infants also begin to display head lag. At one year of age, macrocephaly is often evident as well as a severe motor impairment. At later stages infants will develop optic atrophy and hypotonicity limb stiffness, which then leads to spasticity. With age the symptoms worsen to include seizures and an inability to move voluntarily or even swallow. Death often occurs before adolescence, however in some mild cases of the disease it has been known for some to live into their twenties (Adachi et al., 1973; Hogan and Richardson, 1965; Pratt, 1972).

It was first reported by Kvittengen that increased NAA levels are linked to patients with progressive demyelinising cerebral atrophy. This is due to an increase in the levels of NAA excreted in their urine (Kvittingen et al., 1986). He theorised that the increased NAA excretion could be because of an increased synthesis or reduced degradation of NAA leading to infantile leukodystrophy. It was noted at this point by Hagenfeldt (Hagenfeldt et al., 1987) that NAA aciduria was observed alongside a deficiency of aspartoacylase (ASPA) in the skin fibroblasts of a child with a severe form of leukodystrophy. This led to the proposal that the observed dysmyelination in the CNS was due to a failure of NAA to serve as an acetate carrier of acetyl groups from the mitochondria to the cytoplasm for lipogenesis to occur. However at this point NAA aciduria and ASPA deficiencies were not linked to Canavan's disease. It was only with the work by (Matalon et al., 1988) that the link was made using work in three children with Canavan's disease showing high NAA levels in urine and a lack of ASPA activity in skin fibroblasts. He proposed three possible mechanisms wherein a lack of ASPA activity could lead to the spongy degeneration and demyelisation observed in patients with Canavan's disease. Firstly a high level

of NAA in the brain may directly lead to myelin damage and spongy degeneration. Secondly NAA had been shown to be involved in the production of cerebronic acid and a deficit in myelination which could be responsible for the progression of the leukodystrophy (Shigematsu et al., 1983). Thirdly aspartate can act as a neurotransmitter and that the lack of deacetylase activity might lead to a disruption of aspartate neurotransmission, resulting in neurological disturbances (Bradford and Thomas, 1969). Genetic analysis of Canavan's disease by cloning of the ASPA gene by (Kaul et al., 1993) showed the disease is an autosomal recessive genetic disorder. The changes in NAA in Canavan's disease and the association with myelin damage have provided evidence as to the role of NAA and provided the platform for its assessment in other disease state. One of these disease states is multiple sclerosis. If NAA plays a role in myelin function then it was thought that it would be a useful marker for assessing pathology in multiple sclerosis.

1.4.2 NAA in Multiple Sclerosis

Multiple Sclerosis (MS) is an autoimmune inflammatory demyelinating disease of the CNS which links axonal damage to reduced NAA levels in grey and white matter (Criste and Trapp, 2006). Clinical studies as well as MRS in MS have shown decreased NAA levels associated with the disease's progression in patients (Bruhn et al., 1992; Caramanos et al., 2005; Davie et al., 1994; Larsson et al., 1991; Leary et al., 1999) as well as reduced amounts of NAA and NAA/Cr in MR-visible lesions and in normal appearing white matter (De Stefano et al., 2001; Fu et al., 1998; Tedeschi et al., 2002). NAA reductions in MS patients have also been reported in grey matter and these deficits are thought to be related to the cognitive deficits seen

(Staffen et al., 2005). Further evidence in support of this comes from whole brain studies of NAA, using MRI, that have shown that the cognitive impairment in MS associates with reductions in NAA content (Mathiesen et al., 2006). While atrophy and loss of NAA in the brain are both features of MS, the degree of loss of whole-brain NAA exceeds the development of atrophy by several fold. This leads to the conclusion that neuronal dysfunction may precede any tissue loss seen in MS. Axonal injury begins early in multiple sclerosis (De Stefano et al., 2002) and cumulative axon loss results in progressive disability. However, MS often goes through phases of remission and relapse, and white matter plaques visible in MRS can wane with remission of symptoms (Moffett et al., 2007). MRS studies have shown that NAA levels can be associated with neuronal dysfunction or death since levels of NAA have been seen to recover when MRI visible plaques resolve (Arnold et al., 1990). Patients treated with interferon beta-1b have also reported partial recovery (Narayanan et al., 2001) as well as with glatiramer acetate (Khan et al., 2005) or fluoxetine (Mostert et al., 2006). This work suggests that NAA levels reflect, not only neuronal and axonal integrity, but also may reflect improvements in neuronal energetics and possibly remyelination. Taken together these findings highlight the usefulness of NAA in assessing neuronal function in the brain in MS and monitoring the disease progression over time, along with the possibility of assessing treatment effects. The association of both Canavan's disease and MS with myelin and white matter abnormalities have further strengthened its use as a marker of neuronal dysfunction. These studies have led the way for the investigation of NAA in a number of other disorders, some of which are briefly described below.

1.4.3 NAA in Traumatic Brain Injury

Although the use of CT and MRI scans after Traumatic Brain Injury (TBI) are useful in the management of clinical problems, it was found that the results do not always associate well with the neurological deficit in the early stages after TBI, with even less certainty around the results for long term outcome of the patients (Danielsen, 1999). However, NAA has been seen to be a more sensitive measure of neuronal integrity, damage or death after a TBI (Danielsen, 1999). The use of MRS to determine the NAA content in specific locations in relevance to injury has proved to be of considerable value in the clinical setting (Brooks et al., 2001) using the hypothesis that in white matter NAA is linked to diffuse axonal injury, and in grey matter the loss/dysfunction of neurons. MRS observations in humans after TBI or hypoxia have shown that a time course of hours or days for substantial loss of NAA in the affected region of the brain tissue is observed. Animal studies have seen even sharper loss in NAA levels which have been paralleled with measured decreases in ATP levels, highlighting the potential link between NAA and neuronal energetics. Work done by (Signoretti et al., 2001) used HPLC analysis of brain extracts to show significant and concomitant drops in NAA and ATP within 10 minutes of TBI and a partial recovery in both after 5 days in less serious injuries. In more severe TBI injuries, especially those exacerbated by hypoxia-hypertension, recovery of NAA and ATP levels were not observed. The lowest levels of NAA were observed 5 days after severe TBI. These findings support the idea that NAA levels are linked closely with ATP levels, both of which can recover in brain tissue that has not received substantial, permanent damage. Taken together, these findings demonstrate that NAA measurements are a useful tool for assessing the degree and damage of brain tissue after TBI.

NAA is also a factor in Schizophrenia and Alzheimer's disease and these disorders will be addressed in the subsequent relevant chapter introductions. With the knowledge that NAA is varied in different diseases and can be measured both post-mortem and *in vivo* it is a powerful analytical tool. We hope to use this tool to measure the underlying pathology in different preclinical and clinical samples where we have observed cognitive (behavioural) deficits. Our overall aim is to measure theorized NAA level changes in diseases and hopefully provide a better way of investigating neurological diseases.

1.5 Aims

- Provide a comprehensive analysis of NAA as a biomarker of neuronal function in a preclinical model (NMDA receptor antagonist model) of relevance to schizophrenia.

- Assess the potential of NAA as a biomarker of treatment efficacy in terms of reversing underlying pathological deficits in a preclinical model (NMDA receptor antagonist model) of relevance to schizophrenia.

- Assess the association between *in vivo* (MRS) and *ex vivo* (HPLC analysis) NAA levels in the brains

- Investigate levels of NAA in post-mortem tissue from controls and patients with Schizophrenia in both white and grey matter,

- Investigate levels of NAA in a preclinical model (administration of amyloid beta) of relevance to Alzheimer's Disease.

Chapter 2

Investigating N-acetylaspartate levels in a preclinical model (NMDA receptor antagonist model) of relevance to schizophrenia

2.1 Introduction

2.1.1 General Introduction to Schizophrenia

Schizophrenia was first described by the German psychiatrist Emil Kraepelin in the late nineteenth century as a dementia praecox (Krapelin, 1913). It was, however, then later named "Schizophrenia" by the Swiss psychiatrist Eugen Bleuler as it was realised that it was not a form of dementia but something very different (Bleuler, 1950). The word schizophrenia coming from the Greek meaning, "splitting of the mind", not to be mistaken with split personality disorder but a splitting of mental functions.

The incidence of schizophrenia is about 0.20/1000/year (Messias et al., 2007) and is currently seen in approximately 1% of the world's current population (Current population figures would suggest 70,000,000 worldwide). The onset of schizophrenia is varied. It has been seen that the onset of negative symptoms tends to occur approximately five years before the initial psychotic episode, with onset of positive symptoms closer to the first episode of psychosis (Hafner, 2000).

The age of onset differs between the sexes, where males seem to have a younger onset (Munk-Jorgensen, 1987). The peak age of onset is between the ages of 15 - 24, with males having a higher level of onset than females. Females show a second peak between the ages of 55-64. It is thought that it is the protective anti-dopaminergic effect of oestrogens in women that are the cause in the differences between males and females in ages of onset (Salokangas et al., 2003). It is seen

though that males have an overall higher lifetime risk of developing schizophrenia of about 30-40% when compared with females (Aleman et al., 2003), (McGrath et al., 2004).

The symptoms were initially divided into the positive and negative domains however in later years have been categorised into several distinct types as seen in table 1 (Stahl, 2008).

Positive Symptoms	Delusions, hallucinations and disorganised speech.
Negative Symptoms	Decreased emotional responses, decreased motivation, decreased interests, decreased thought and speech, decreased pleasure response.
Cognitive Deficits	Attention, working and verbal memory and executive function.
Mood (General) Symptoms	Depression, anxiety, hostility, aggression and suicide.

Table 1: Unique set of symptoms displayed by a schizophrenic patient (Kay et al., 1987).

Classical and atypical antipsychotics currently available only have an effect on positive and some negative symptoms. There is a current unmet clinical need for treatment of the main negative and cognitive symptoms in schizophrenia.

2.1.2 Cognition in Schizophrenia

Cognitive function relates to a patient's ability to process and use information around them. Deficits seen in cognition, in patients with schizophrenia, cause the most

problems with a schizophrenic patient's re-integration into society and are one of the central features that determine recovery in schizophrenia (Goldberg et al., 1995). The collaboration between the National institute of Mental Health, the University of California, Los Angeles, and the United States Food and Drug Administration termed – Measurement and Treatment Research to Improve Cognition in Schizophrenia (MATRICS) initiative (Marder et al., 2004) was formed to better identify the cognitive domains of schizophrenia. It was MATRICS that used the identification of the seven domains of cognition and created a standardised set of cognitive testing batteries for patients with schizophrenia that is consistent and can be comparable across numerous studies (Marder and Fenton, 2004). As such cognition in schizophrenia was defined using these seven cognitive domains, attention/vigilance, working memory, reasoning and problem solving, processing speed, visual learning and memory, verbal learning and memory, and social cognition (Nuechterlein et al., 2004) as these are the cognitive domains consistently showing as a problem in schizophrenia.

The MATRICS testing battery contains ten tests that assess the seven cognitive domains mentioned above. Table 2 shows the specific cognitive domain and which test models the specific cognitive domain in both human and animal studies.

Cognitive Domain	Animal Models/Tests	MATRICS Clinical Battery
Working memory	Operant or T-maze, Radial arm maze	BACS WMS-III Spatial Span WAIS-III Letter-Number sequence
Attention/vigilance (pre-attentive processing)	5-Choice Serial Reaction Time Task *PPI, auditory gating*	3-7 CPT Identical pairs CPT *PPI, auditory gating*
Verbal learning and memory		NAB- Daily Living Memory
Visual learning & memory	Novel Object Recognition	NAB – Shape Learning
Speed of processing	5-Choice Serial Reaction Simple Reaction time tasks	Category fluency Trail making A
Reasoning & problem solving	Attentional set shifting Maze tasks Serial Reversal Learning	WAIS-III Block design BACS- Tower of London NAB - Mazes
Social cognition	Social interaction/Social recognition?	MSCEIT – Managing emotions

Table 2: MATRICS Test Battery - Overview of preclinical rodent tasks which map onto the MATRICS test battery. (http://www.matrics.ucla.edu) Highlighted text in the table relates to behavioural tests that will be used in this thesis.

The dopamine hypothesis is useful for study of the positive symptoms however does not account for the negative and cognitive symptoms; cognitive deficits are more related to the GABA/glutamate transmission pathways. The glutamate hypothesis has become one of the main theories to explain these deficits. Since the cognitive deficits are one of the main factors preventing patients' outcome and re-integration the glutamate hypothesis has been explored and tested with the aim of developing novel targets for these symptom domains.

2.1.3 Glutamate Hypothesis

Glutamate is the most abundant excitatory neurotransmitter in the mammalian brain. Glutamate receptors can be divided into two distinct groups, ionotropic and metabotropic (Hollmann and Heinemann, 1994; Nakanishi, 1992; Seeburg, 1993). The ionotrophic receptors contain cation-specific ion channels and can be further divided into three groups; α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), kainate and N-methyl-D-aspartate (NMDA) receptor channels. The main glutamate hypothesis arises from the NMDA receptor hypofunction hypothesis of schizophrenia (Olney and Farber, 1995a, b). It's postulated that NMDA hypofunction produces a reduction in inhibitory firing mediated by GABAergic neurotransmission.

The first origins of the glutamate hypothesis date back to the 1950's when it was first shown that the anaesthetic phencyclidine (PCP) was seen to induce psychotic symptoms in a human patient (Luby et al., 1959). This work was further substantiated with work demonstrating similar effects with ketamine and MK-801 (Krystal et al., 1994; Shulgin, 1964). It was the work by Krystal and Shulgin, along with the general realisation that DA alone, could not be accountable for both the negative and cognitive symptoms of schizophrenia. In support of this hypothesis it has also been shown that glycine administration enhances NMDA receptor function which can improve schizophrenic symptoms when used in conjunction with other antipsychotic treatment (Rosse et al., 1989).

We understand the underlying theories behind the deficits in schizophrenia. It is important to understand the application to an animal model so that we can create a more relevant and reliable model for both pharmacological and physiological testing.

2.1.4 Animal Models of Schizophrenia

The development of successful animal models allows us to be able to achieve a better understanding of the disease state and its underlying pathology along with providing a platform for testing of novel compounds. Ultimately animal models of schizophrenia aim to:

- mimic the fundamental symptoms found in a schizophrenic patient e.g. cognitive deficits (*face validity*).
- conform to a theoretical rationale, such as the proposed pathophysiology and symptomatology in schizophrenia (*construct validity*).
- possess the ability to predict known and novel therapeutics (*predictive validity*) (Floresco et al., 2005; Hagan and Jones, 2005).

The concept of etiological validity is closely related to construct validity. For example a model has etiological validity if the etiologies of the cognitive deficit in the animal model and the human condition are similar. The MATRICS (http://www.matrics.ucla.edu) testing battery allows for a good cross comparison from rodent tasks onto human cognitive domains (see table 2).

2.1.5 Pharmacological Models of Schizophrenia

As mentioned above, drugs that cause schizophrenic like psychotic symptoms in humans can be used to create a pharmacological model of schizophrenia. These include amphetamine, which causes more florid positive symptoms of schizophrenia such as paranoia, hallucinations and delusions. This is modelled as hyperactivity (Jentsch et al., 1998b), but such an approach has limited value when it comes to mimicking other aspects of the disease, as this approach is mainly based around the neurotransmitter dopamine and its role in the positive symptoms.

Other pharmacological approaches include administration of NMDA receptor antagonists (e.g. phencyclidine (PCP), ketamine or MK-801). It has been seen that NMDA receptor antagonist models are particularly good at mimicking a broader range of behavioural and pathological symptoms seen in schizophrenia and these models have become one of the most reliable preclinical models of schizophrenia (Jentsch and Roth, 1999) Neill et al., 2010). Evidence for the validity of the models comes from comparing effects in humans, primates and rodents. Table 3 highlights some of the behavioural effects in relation to symptom domains seen following both acute and chronic exposure to NMDA receptor antagonists in clinical and preclinical situations.

	Acute Exposure	Chronic Exposure
Human Studies		
Psychosis	Intense (Hours)	Intense (Days to Weeks)
Hallucinations	Visual Illusions	Auditory and paranoid (Days to Weeks)
Delusions	Yes (Hours)	Frequently religious (Days to Weeks)
Thought Disorder	Yes (Hours)	Yes (Days to Weeks)
Affect	Euphoric to catatonic (Hours)	Anxious, labile or paranoid (Days to Weeks)
Cognition	Impaired (Transiently)	Impaired (Persistently)
Frontal Blood Flow	Impaired (Transiently)	Decreased (Persistently)
	Acute Exposure	Chronic Exposure
Rodent Studies		
Sensorimotor Gating	Impaired	Preserved
Motor Function	Impaired	Preserved
Motivation	Impaired	Preserved
Associative Processes	Impaired	Preserved
Social Behaviour	Reduced	Reduced
Locomotion	Impaired	Augmented response to stress or amphetamine
Frontal Blood Flow	Impaired (Transiently)	Decreased (Persistently)

Table 3: NMDA receptor antagonists effects
Differing psychiatric and biological effects on acute versus long-term exposure to NMDA receptor antagonists on human and rodent behaviour. (Jentsch and Roth, 1999)

One key aspect is the dosing schedule used to induce the deficits and the time after dosing that the testing takes place. It is clear from the tables above that there are a number of differences observed when looking at acute versus chronic exposure. In terms of predictive and pathological validity, a sub chronic PCP exposure model is considered to be one of the most effective methods of inducing cognitive and

pathological (e.g. deficits in parvalbumin containing interneurons in the prefrontal cortex and hippocampus) deficits similar to those observed in the clinic (Neill et al., 2010). This approach allows us to investigate the effects of novel treatment strategies on both the behavioural and pathological deficits of relevance to the illness.

2.1.6 Aims

In this chapter we aim to provide a comprehensive analysis of NAA as a biomarker of neuronal dysfunction in a preclinical model (NMDA receptor antagonist model) of relevance to schizophrenia.

To achieve this we will:

- Investigate levels of NAA from post-mortem tissue in vehicle and sub-chronic PCP treated animals.
- Investigate the neuronal associates of NAA deficits in regions where we find changes.
- Assess the potential of NAA as a biomarker of treatment efficacy in terms of reversing underlying pathological deficits.

2.2 Materials and Methods

2.2.1 Animals

All studies used adult female (240-280g) hooded-Lister rats (Charles River or Harlan, UK). Rats were housed (4-5 per cage) in solid floored plastic cages (38 x 59 x 24 cm) containing sawdust, paper sizzle nest (Datesand, Ltd., Manchester, England) and fun tunnels (plastic environmental enrichment tubes., Datesand, Ltd., Manchester, England). Food (Special Diet Services Ltd., Essex, England) and drinking water was available *ad-libitum* in the home cage. The rats were disturbed only for behavioural testing and cleaning which consisted of changing the cage twice per week, and experimental procedures. Rats were housed in a single sex colony which was maintained under a constant temperature of approximately 21 ± 1 °C and humidity (40-50%) under a 12h light:dark cycle (lights on at 0700 h). All experiments were conducted during the light phase and were carried out between 0900 h and 1630 h. All studies were compliant with the Animal Scientific Procedures act (1986) and approved by the University of Bradford Ethical Review Process.

In this chapter three cohorts of animals were used:

Cohort	Groups	Experiment
1	Vehicle (n=8) PCP (n=8)	Investigating NAA in the brains of Vehicle and PCP treated animals
2	Vehicle (n=6) PCP (n=6)	Investigating neuronal cell number in the Frontal Cortex of Vehicle and PCP treated animals
3	Vehicle (n=7) PCP (n=5) PCP + Olanzapine (n=5)	Investigating NAA in the Frontal Cortex of Vehicle, PCP alone and PCP + Chronic Olanzapine treated animals

Table 4: Animal cohorts

2.2.2 Drug Treatment

PCP and olanzapine were purchased from Sigma-Aldrich UK. Drugs were stored in a dark sealed container in a locked drug cabinet before use. PCP was made in 250 ml batches and stored in a designated locked fridge. Olanzapine solution was made fresh each day before administration.

2.2.3 Sub Chronic PCP and Olanzapine dosing regimen

The sub-chronic PCP dosing regimen was adapted from earlier studies by (Jentsch and Roth, 1999) and has been shown to produce enduring behavioural, neurochemical and pathological changes associated with schizophrenia (Fletcher et al., 2005). Sub-chronic PCP was administered via the intraperitoneal (i.p.) route in a volume of 1 ml/kg at 2 mg/kg, calculated as the base equivalent weight, twice per day for 7-days. Treatment with vehicle or PCP was followed by a 1-week washout

period prior to testing. The 1-week washout period following sub-chronic PCP treatment is necessary to prevent the behaviour of the rats being influenced either by direct drug effects or by drug withdrawal effects (Jentsch et al., 1998a). Similar to the PCP dosing regimen Olanzapine was administered via the i.p. route in a volume of 1 ml/kg at 1.5 mg/kg. However, Olanzapine was only administered once daily for 28 days. At the end of dosing the rats were immediately terminated and the brains collected. The summary time scale for the PCP and olanzapine dosing regimen is shown in figure 4.

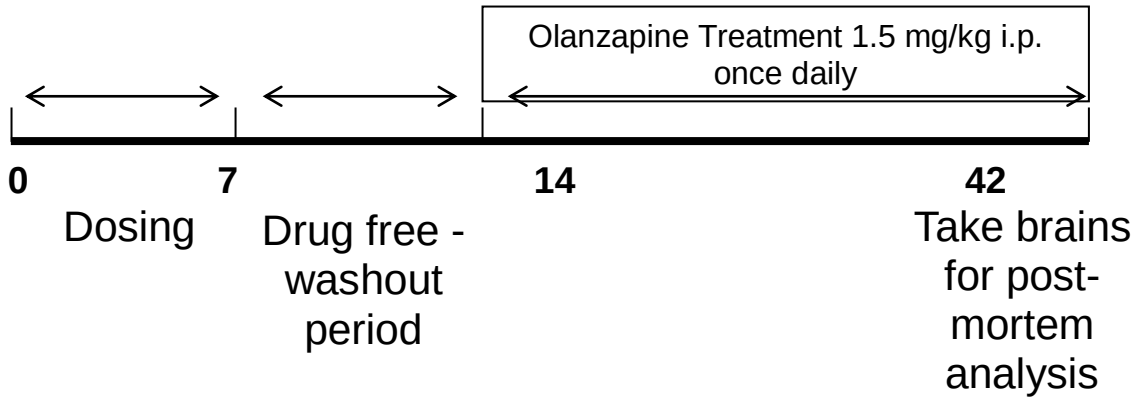

Figure 4: Dosing regimen used for the studies.

2.2.4 Brain Dissection and sample preparation

At the end of the experiments animals from cohort 1 & 3 were sacrificed by CO_2 inhalation. Brains were quickly removed and the frontal cortex (anterior to bregma +3.20), striatum (bregma +1.20 to -0.20), hippocampus (dorsal: between bregma -2.30 to -3.80; ventral: bregma -4.8 to -6.04) and temporal cortex (bregma -2.30 to -3.80) were collected ((Paxinos and Watson, 1998) See figure 5). The analysis included bilateral hemispheric dissection. Dissected tissues were immediately frozen on dry ice and stored at -80 for further analysis.

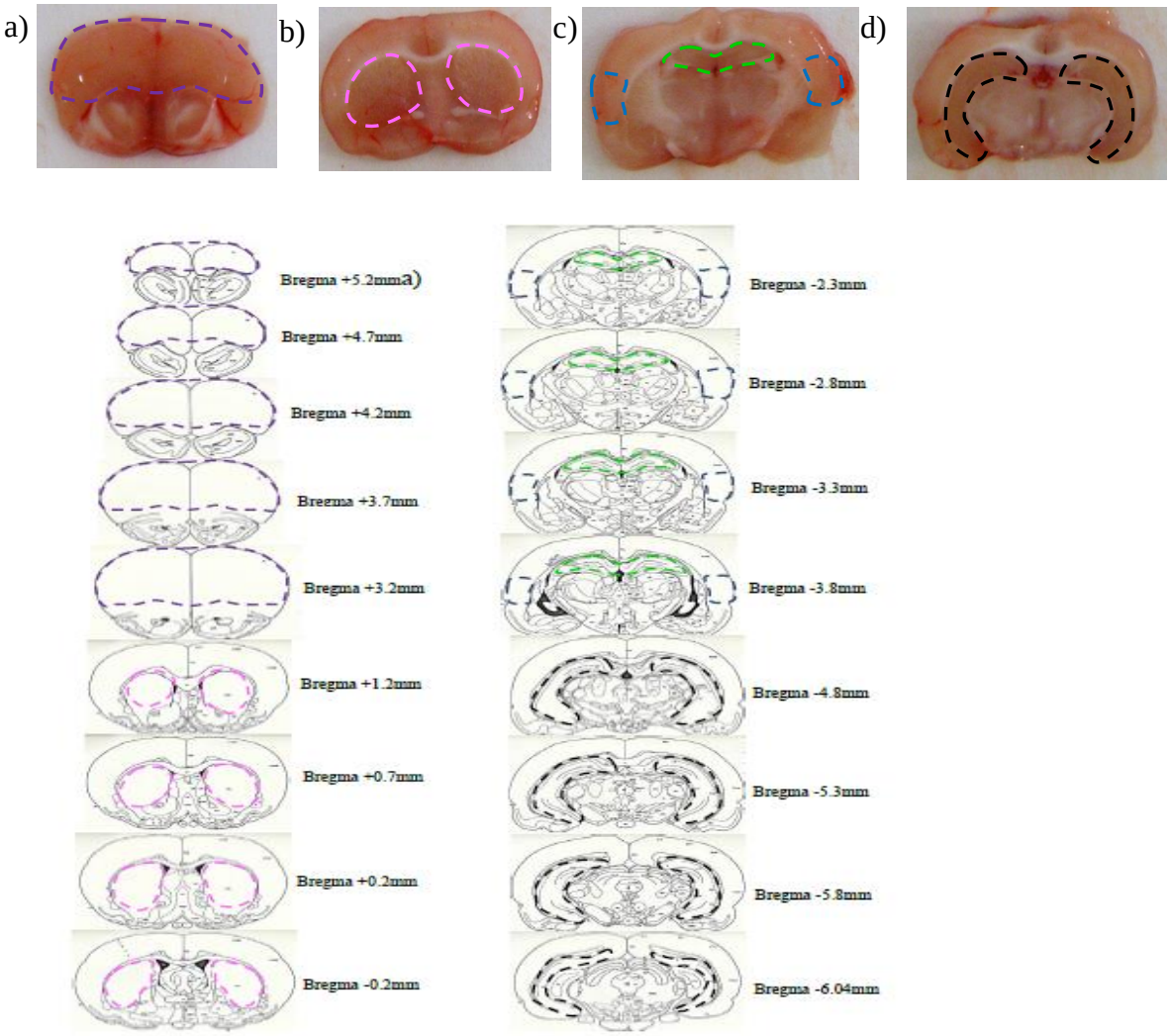

Figure 5: Rat brain sections

Sections taken from the rat brain. Dashed line represents place of dissection, where a) frontal cortex (violet line); b) striatum (pink line); c) dorsal hippocampus (green line) and temporal cortex (blue line); d) ventral hippocampus (black line). Tissues were dissected from both, left and right hemisphere and analysed together.

Frozen sections were then weighed and 10 times their volume of 0.1 M perchloric acid was added. Tissues were homogenised and centrifuged for 5 min at 12,000 *x g* to form a pellet (Eppendorf Centrifuge 5415 C). The supernatant was removed and

used to prepare the samples for analysis. Samples consisted of 50 µL of supernatant added to 920 µL of 50 mM phosphate buffer and 30µL sodium carbonate. Samples were extracted using anion exchange columns (Figure 5).

2.2.5 Column Preparation

Columns were washed and prepared to condition the silica surface of the columns. Washing and preparation of the columns decreases the ionic strength and achieves maximum retention for NAA. Copy of solution make up and standard protocols are found in Appendix A of this thesis.

Running order for column preparation and extraction:

1ml Vol, SAX, Ion exchange, 100 mg/1 ml

Run through the columns in the following order:

[Run all to waste]

1. 1x 1 ml Methanol
2. 2x 1 ml 0.5% Phosphoric Acid
3. 4x 1 ml 1M Phosphate Buffer pH6
4. 2x 1 ml 50mM Phosphate Buffer pH6
5. Add sample (1 ml) and run to waste
6. 2x 1 ml 2 mM Phosphate Buffer pH6

[COLLECT IN 1.5 ml EPPENDORFS]

7. Elute samples with 1.5 ml 0.5% Phosphoric Acid

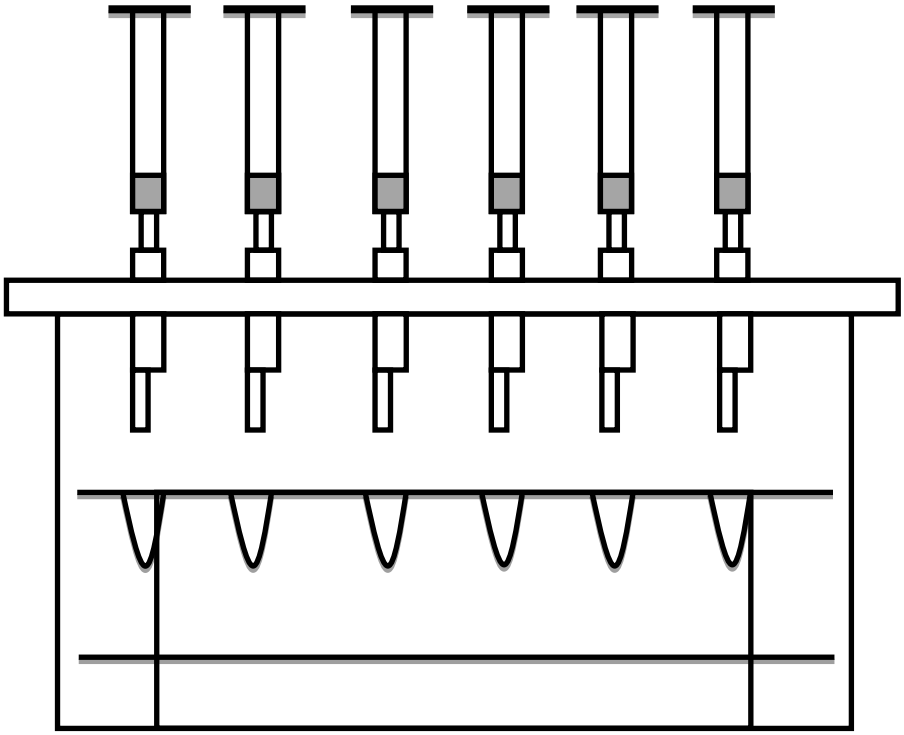

Figure 6: Layout of Ion Exchange apparatus.

Columns are washed to waste and then 1.5 ml Eppendorfs are used for collection at the elution stage of collection.

After collection, samples were analysed by high performance liquid chromatography with ultraviolet (UV) detection. Un-extracted and extracted standard solutions were prepared to evaluate potential loss of the sample and define analyte concentration. The un-extracted NAA standard consisted of 10-50 µl of 1 mM NAA and was made up to 1.5 ml with 0.5% phosphoric acid. The extracted NAA standard was created by the addition of 10-50 µl of 1mM NAA and 990-950 µl of 50 mM phosphate buffer (pH 6).

2.2.6 HPLC Determination of NAA

Collected samples were injected (50 µl) onto a reversed phase octadecyl column (C18) with particle size 4µm (150 x 4.6 mm) using a SIL-20AC refrigerated (4°C) autosampler. The temperature of the column was maintained at 40°C to preserve the flow of the mobile phase (LC-22 Temperature Controller). Mobile phase comprised of 0.1% phosphoric acid at pH 2.3 and was pumped at flow rate 0.5 ml/min using Series 200 micro ic pump. UV detector (Waters 486 Tunable Absorbance detector) was set at sensitivity 0.01 and wavelength 215 nm.

A control experiment was conducted with un-extracted and extracted known amounts of NAA standards. During sample analysis, analyte was clearly separated from the other components and identified by the retention time when compared to standards. Peak heights were measured and the percentage of the recovery was calculated. Therefore, measurements were corrected depending on the loss of analyte due to extraction.

2.2.7 HPLC Equipment

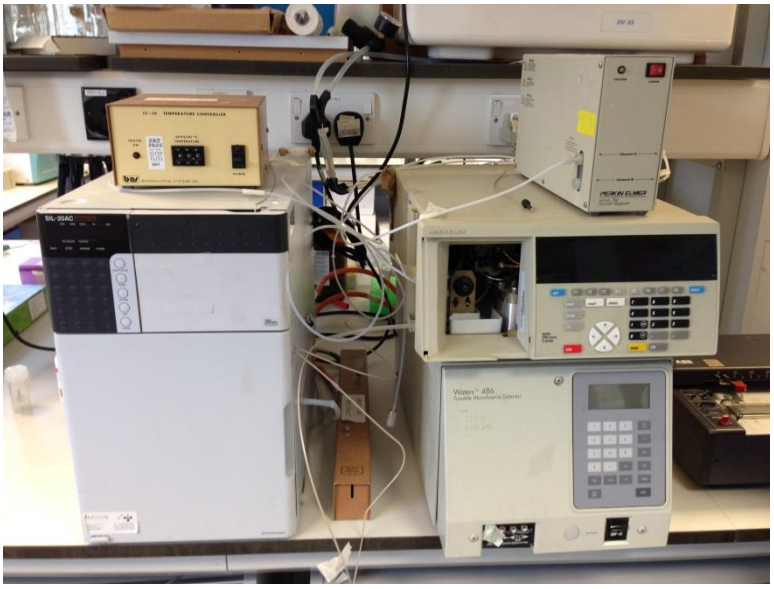

Figure 7: HPLC equipment setup

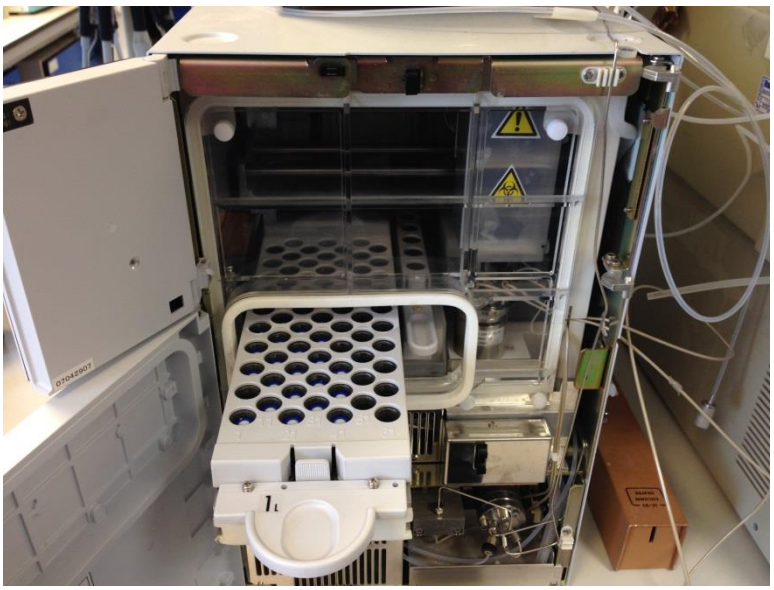

Figure 8: Auto sampler with sample rack exposed

Figure 9: HPLC pump, de-gasser and absorbance detector

Figure 10: Chart recorder

Quantification of NAA was achieved by the following process. Firstly the sample peak height must be calculated with the following formula:

C*f (Correction factor)* = un-extracted peak height / extracted peak height

Sample peak height was then corrected to absolute values (A*v*) by dividing the sample peak height by C*f*. A standard linear curve must be drawn using the un-extracted standards with a linear trend line to 0,0 and calculate y = ax from the trend line. Quantity of NAA in injected sample volume (50 µl) was calculated using the following formula:

S*c* (NAA Sample content, ng) = A*v* / a

Brain tissue concentration was calculated using this final equation:

Q_{NAA}(Quantity of NAA) = S*c* x 6

S*c* is multiplied by 6 as all volumes are normalised. Tissue is diluted in 10 times volume of perchloric acid Q_{NAA}/10 (ng/µl). Of this sample 50 µl is taken for analysis so 50 x Q_{NAA}/10 = Q_{NAA} placed in 1 ml then extracted in SAX columns to 1.5 ml. With just 50 µl added to the HPLC this means the concentration is 50/1500 or 1/30. Therefore 5Q_{NAA} *1/30 = Q_{NAA}/6 in 50 µl. Therefore this is the S*c* which presents S*c* = Q_{NAA} / 6 or Q_{NAA} (ng) = 6 x S*c* in 1 mg of tissue.

Levels of NAA were recorded as ng NAA / mg tissue and presented as mean ± standard error mean (SEM).

2.2.8 Brain Dissection and sample preparation

At the end of the experiments animals (Cohort 2) were sacrificed by CO_2 inhalation. Brains were removed and fixed in 10% phosphate buffered formalin for 3 days, before being embedded in wax. Six sections (10 μm) from the frontal cortex determined using a rat brain atlas (Paxinos and Watson, 1998) were mounted onto slides coated with 3-aminopropyltriethoxysilane (APES) (Sigma, UK) and stained with cresyl violet. Briefly, following clearing in xylene and rehydration in graded alcohol, sections were incubated in 0.01% (v/w) cresyl violet solution for 15 minutes. Following dehydration in graded alcohol coverslips were mounted with DPX solution and left to dry. All slides were coded and analysed blind to treatment. Stained sections were then scanned at 4× magnification using an Olympus microscope interfaced to an Image ProPlus analysis system (USA) via a JVC 3-CCD video camera. Estimations of neuronal density (cells/mm^2) were carried out, in six coronal sections (60 μm apart).

2.2.9 Statistical Analysis

Determination of the deficits in regional NAA concentrations and the effect of chronic olanzapine in vehicle and sub-chronic PCP treated rats was evaluated by the analysis of variance (ANOVA) (Casanova et al.). Determination in the deficits of neuronal associates in NAA deficits were evaluated using Student's t-test. The statistical analysis was completed on SPSS/PASW version 19. A p value < 0.05 was considered to be significant.

2.3 Results

2.3.1 Validation

Control experiment of extracted and un-extracted NAA standards run on HPLC. Extracted being standard run on extraction columns, unextracted directly run on HPLC with no extraction columns used.

Standard Solution	Peak Height (mm)		
	10 µM	20 µM	30 µM
Un-extracted Standard	65	120	185
Extracted Standard	47	85	128
% Recovery	72.3	70.83	69.19

Table 5: NAA Standard Validation

Peak height measurements (mm) of known amount of NAA in the un-extracted and extracted standards at concentrations: 10, 20, 30 µM. The recovery was calculated according to the relative loss.

Recovery of NAA was found to be consistent and independent of higher concentrations of standard. Identification of NAA Figure 11 shows an example of an HPLC chromatogram used in the validation study. It is important to notice that the control demonstrates only a pressure wave peak and does not indicate any other components. A visible effect for the loss of the sample due to the extraction can be observed when comparing peaks of standards. There is a considerably lower peak in the extracted standard compared to that of the un-extracted standard. The retention time of NAA was determined at 7.0 minutes when compared to external standards. Run time was set to be 15 minutes as it allowed a full extraction of NAA and other analyses in brain samples.

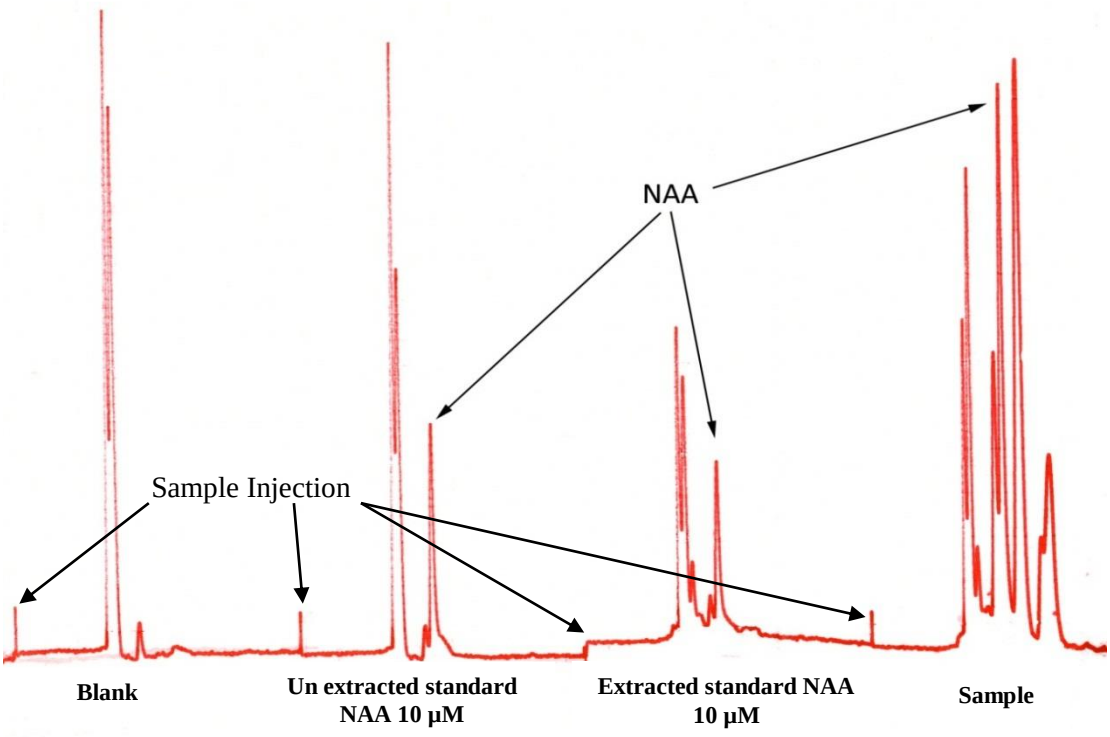

Figure 11: An example of the HPLC chromatogram.

UV detection wavelength set at 215 nm and sensitivity at 0.01. The graph represents outlines of blank, unextracted and extracted 10µM NAA standards and sample 1 from ventral hippocampus (VH1 - Far right peak group).

2.3.2 Investigating the levels of NAA in vehicle and sub-chronic phencyclidine treated animals

NAA levels varied between the different brain regions. The lowest detected levels were in the dorsal hippocampus (Vehicle, 983.8 ng/g, PCP, 1039.0 ng/g) and the highest levels seen in the ventral hippocampus (Vehicle, 1321.6 ng/mg, PCP, 1209.1 ng/mg).

Analysis of the data with ANOVA showed that treatment with sub-chronic PCP produced a significant reduction of NAA in the frontal cortex ($F_{1,15}$=8.83, P<0.05; figure 12) and the ventral hippocampus ($F_{1,15}$=5.241, P<0.05; figure 12). Significant deficits were not seen in the striatum ($F_{1,15}$=1.058, NS; figure 12), dorsal hippocampus ($F_{1,15}$=0.412, NS; figure 12) and temporal cortex ($F_{1,15}$=2.197, NS; figure 12).

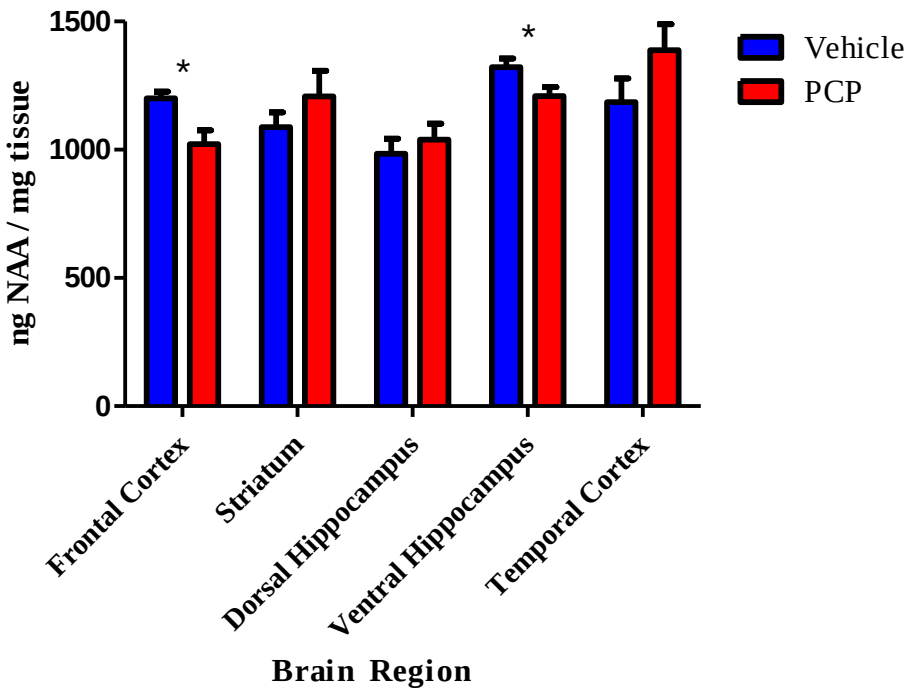

Figure 12: N-acetylaspartate (ng / mg) in the frontal cortex, striatum, dorsal, ventral hippocampus and temporal cortex from rats treated with vehicle (0.9% saline i.p.) or phencyclidine (2.0 mg/kg, i.p.) (n= 8 per group). Data is shown as mean ± SEM. * P < 0.05; significant difference between Vehicle and PCP groups in Frontal Cortex and Ventral Hippocampus brain regions.

2.3.3 Investigating the effects of chronic olanzapine treatment on NAA levels in the frontal cortex of vehicle and sub-chronic phencyclidine treated animals

Analysis of the data with ANOVA showed a significant ($F_{2, 16}$=4.80, P<0.05; figure 13) difference between the groups. Following planned post hoc LSD t-test significant (P<0.05) reduction in the levels of NAA was seen in the PCP and Olanzapine groups when compared to vehicle.

	NAA Peak Height (mm)	Peak Height Corrected	NAA in Injected Sample	Brain Tissue Concentration (ng NAA / mg tissue)
Vehicle	79	80	260	1558
	70	71	230	1380
	82	83	270	1617
	66	67	217	1302
	79	80	260	1558
	100	102	329	1972
	68	69	224	1341
PCP	69	70	227	1361
	53	54	174	1045
	58	59	191	1144
	76	77	250	1499
	58	59	191	1144
Olanzapine	63	64	207	1242
	60	61	197	1183
	67	68	220	1321
	68	69	224	1341
	64	65	210	1262

Table 6: Cohort three data shows that olanzapine has no effect on reversal of NAA levels when compared to vehicle. These data show that olanzapine has the same significant (P<0.05) reduction as the PCP group.

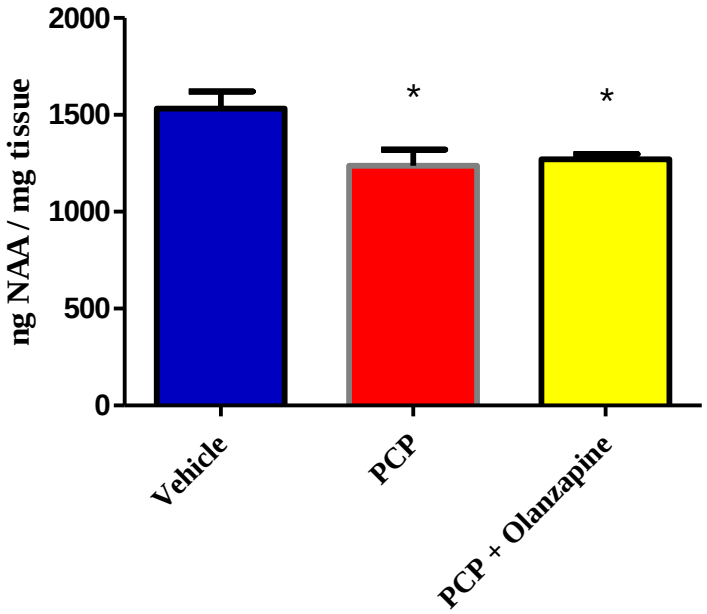

Figure 13: Concentration of N-acetylaspartate (ng NAA / mg tissue) in the frontal cortex from rats treated with phencyclidine injections (2.0 mg/kg, i.p.), Saline (0.9%, i.p.) in control group or olanzapine (1.5 mg/kg, i.p.) once daily for 28 days. (n= 5-7). Data is shown as mean ± SEM. * P < 0.05; significant difference in PCP and PCP + Olanzapine groups when compared with Vehicle.

2.3.4 Investigate the neuronal associates of NAA deficits in the frontal cortex of vehicle and sub-chronic phencyclidine treated animals

Stained sections were scanned at 4x magnification (Figure 14a). Cell count was determined in the frontal cortex using unbiased 2D stereological examination of 6 sections per animals. Stained cells were found throughout the frontal cortex and counted at 40x magnification using randomly generated counting frames (60 µm x 60 µm) that included consistent inclusion / exclusion lines. Any stained cells

touching the red (exclusion) lines were not included in the count. A total of 30 counting frames were analysed per section.

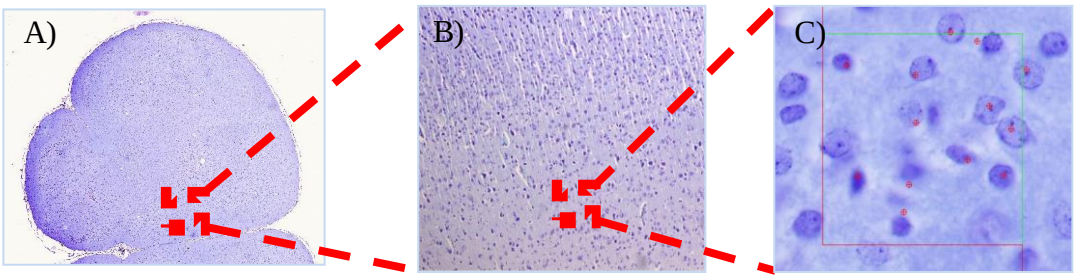

Figure 14: Illustrations showing the section of interest under different magnifications. (A) at 4x magnification, (B) at 10x magnification and (C) at 40x. Included on image C is a counting frame (area of 60µm x 60µm) with the inclusion (green) and exclusion (red) lines.

Raw count result for each individual rat is shown in table 7. The count is averaged for 30 randomly chosen counting frames per section for a total of 6 sections per animal within the region of interest (frontal cortex). The count is then corrected for an mm^2 area and averaged to give a cell count for the entire group.

PCP			**Vehicle**		
RAT	**Count**	**Cells/mm^2**	**RAT**	**Count**	**Cells/mm^2**
2	5.47	1519.44	9	8.12	2255.56
3	6.82	1894.44	12	7.6	2111.11
5	6.39	1775.00	13	7.49	2080.56
6	6.28	1744.44	14	7.57	2102.78
7	5.63	1563.89	15	7.17	1991.67
8	7.38	2050.00	16	7.75	2152.78
Mean	**6.33**	**1757.87**	**Mean**	**7.62**	**2115.74**
SEM	**0.29**	**81.43**	**SEM**	**0.13**	**35.47**
P value	**0.002**				

Table 7: Neuronal cell density in vehicle and PCP treated animals in the Frontal Cortex. The count is a mean taken from a minimum of 8 sections from each animal. Cell density is expressed as cell/mm2 and shown as Mean ± SEM.

We found significant ($F_{1, 11}$=16.23, P<0.01; figure 15) deficits in cell density in the PCP group when compared to vehicle (Table 11 & Figure 15).

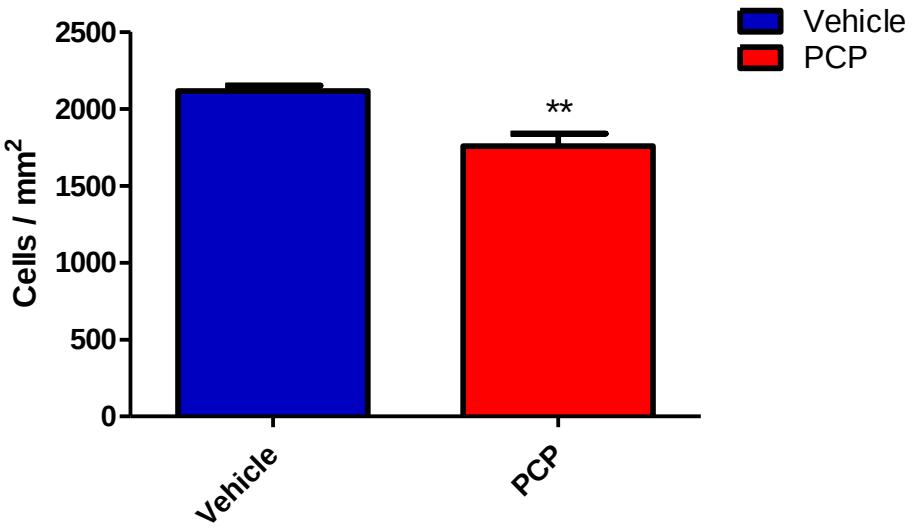

Figure 15. Neuronal cell density in the frontal cortex from rats treated with phencyclidine injections (2.0 mg/kg, i.p.), Saline (0.9%, i.p. (n= 6). Data is shown as mean ± SEM. * P < 0.01; significant difference in PCP group when compared with Vehicle.

2.4 Discussion

In this research, sub chronic phencyclidine administration was used as a tool to examine changes of N-acetylaspartate level in different regions of the rat brain, to determine whether the sub-chronic PCP model could present similarities in pathology of schizophrenia and in turn if NAA could be used to measure these deficits. The results demonstrated that significant deficits can be detected in the frontal cortex and ventral hippocampus but not in the striatum, dorsal hippocampus or temporal cortex. In this chapter we showed that there are significant NAA deficits in a sub-chronic PCP model of schizophrenia in the frontal cortex and the ventral

hippocampus and that chronic olanzapine cannot ameliorate the NAA deficit caused by sub-chronic PCP administration. Then finally that there is a reduction in the cell density in the PCP group when analysed with cresyl violet.

2.4.1 Investigating effects of sub-chronic PCP on NAA levels - Frontal Cortex

We have demonstrated that sub-chronic PCP treatment leads to decreased NAA levels in the frontal cortex (P= 0.010) suggesting neuronal dysfunction. Our finding further validates positive results of other studies using the phencyclidine model (Cochran et al., 2003; Reynolds et al., 2005). In our case, the blockage of NMDA receptors successfully confirms the observed hypofrontality and potential abnormalities in neurons of the frontal lobe by decreased NAA levels. One of the proposed hypothesis is the involvement of NAA in the process of myelin formation (Chakraborty et al., 2001). NAA would act on an acetyl CoA present via NMDA receptors in oligodendrocytes, which were shown to be dysfunctional in the schizophrenia (Tkachev et al., 2003). These findings are important since NAA is a marker for general neuronal function and, therefore, have the potential to measure these levels of hypofrontality. This is supported in this chapter by the findings with cresyl violet demonstrating a reduction in cell count in the PCP treated animals. Both of these markers are general in terms of not highlighting any specific neuronal systems. However, numerous studies report deficits of GABAergic interneurons (parvalbumin immunoreactive). These deficits have been reported in schizophrenic patients (Beasley and Reynolds, 1997) and in the sub-chronic PCP model (Neill et al., 2010). Reductions in GABAergic interneurons have also previously been reported to be reduced in postnatal PCP treated rats (Kjaerby et al., 2014; Kjaerby et

al., 2013), as well as following MK-801 (Abekawa et al., 2007), all of which are considered useful models for mimicking the cognitive dysfunction seen in schizophrenia.

A neuronal dystrophy and a substantial reduction of the frontal lobe have been demonstrated in post-mortem studies of schizophrenic brains (Rajkowska et al., 1998; Selemon et al., 1995). Decreased glucose utilization and atypical connectivity are common findings in neuroimaging studies in humans (Weinberger et al., 1992) as well as in primates following chronic PCP treatment (Jentsch et al., 1997). These deficits have been linked to the severity of negative and cognitive symptoms to the frontal lobe dysfunction (Hazlett et al., 2000; Sigmundsson et al., 2001; Williamson et al., 1991). Consequently, our marker has been an object of interest and studies have frequently reported a significant decrease of the NAA levels in the frontal lobe of patients diagnosed with schizophrenia (Callicott et al., 2000b).

This fact supports the idea that the NMDA receptors blockage reflects schizophrenia pathophysiology in greater detail. Given the changes in NAA levels, there is strong association between frontal cortex abnormalities of schizophrenics and the sub-chronic PCP model. Since a reduction in NAA level has been linked to a decline in cognitive function (Ackl et al., 2005; Jessen et al., 2001; Jessen et al., 2000) a reduction in the frontal cortex supports this previous finding. Cognitive deficits due to this have previously been reported (Neill et al., 2010) from our research group displaying the cognitive dysfunctioning (working memory, object recognition etc.) shown in the PCP model using a variety of cognitive tests.

2.4.2 Olanzapine does not ameliorate the deficit in NAA caused by PCP in the frontal cortex

In this chapter we found that chronic administration with olanzapine was unable to reverse the pathological deficits (i.e. deficits in NAA / deficits in cresyl violet staining). We have previously demonstrated that the cognitive deficits (deficits in reversal learning) induced by sub-chronic PCP were attenuated following chronic olanzapine treatment (following a similar dosing regimen as in the current study) (McLean et al., 2010). This attenuation was present following olanzapine administration. Olanzapine's ability to attenuate the PCP-induced cognitive deficits may be a function of its modulation of dopaminergic and glutamatergic activity through the antagonism of 5-HT receptor subtypes. An antagonism of the 5-HT_{2A} receptors within the prefrontal cortex increases dopamine transmission and consequently alleviates sub-chronic PCP induced dysfunction (Bortolozzi et al., 2010). However, with the data collected in this thesis we have shown that the effect of olanzapine is a short lived effect that only causes a pharmacological change and not a long term neurochemical or physiological change. This finding is supported by the findings by (Elsworth et al., 2011) who shows that olanzapine reverses the loss of asymmetric spine synapses in the PFC of a PCP induced rat model. However, in their study they have only focused on the acute effect of olanzapine and not the chronic, again showing a temporary reversal in PCP deficits and not a long term reversal. Therefore olanzapine has no long term restoration on the neuronal function.

These findings bring light to the limitations of the current treatments available for schizophrenics, in that the beneficial effects on symptoms are present with the drug on board and that the drugs are not demonstrating any long term benefits in terms of tackling the underlying pathology. As such, failure to comply with treatment will result in a rapid decline in patient mental health.

Taken together the findings in this cohort demonstrates, in this case, that NAA does not fit with our hypothesis as a biomarker for treatment efficacy in terms of assessing the ability of novel treatments to target/attenuate the underlying cause of the behavioural/cognitive deficits.

2.4.3 Investigating effects of sub-chronic PCP on NAA levels - Striatum

In contrast, we did not find any significant deficits in the striatum (P= 0.321). This part of the brain has been strongly implicated in the pathology of schizophrenia (Cleghorn et al., 1992). Post-mortem and neuroimaging studies reported increase in striatal tissue volume, but it is believed to be a result of the medication (Chakos et al., 1994). NAA levels show few positive findings, usually only in specific subsections e.g. caudate gyrus (Reynolds and Reynolds, 2011). Investigation of the striatum did not show differences in the chronic PCP model (Reynolds et al., 2005). This region receives multiple projections from the prefrontal cortex and it is mostly composed of dopamine neurones. Phencyclidine action demonstrates the activation of dopamine pathways by the blockage of NMDA receptors (Deutch et al., 1987). It would suggest that direct antagonist drug action has a minimal effect on the striatal

tissue. Testing of NMDA action on striatum was performed in an adult and neonatal model (rat male and female) with no significant changes found (Sircar and Soliman, 2003). Nevertheless, decreased NAA level in the frontal lobe was presented as a marker for the functionality and availability of D2 receptors (Bertolino et al., 1999). Also, NAA levels in rat striatum increased following chronic treatment with the typical antipsychotic haloperidol (Harte et al., 2005).

2.4.4 Investigating effects of sub-chronic PCP on NAA levels - differential effects in the hippocampus

To our knowledge, this is the first time that NMDA receptor antagonist effect on the NAA level was investigated separately in dorsal and ventral hippocampus in the sub-chronic PCP model. We detected significant deficits of NAA in the ventral hippocampus (P= 0.039), but not in the dorsal hippocampus (P= 0.534). The hippocampus is now recognised as a heterogeneous structure, which may act independently. It's role is highly implicated in the pathology of the disease, together with significantly decreased NAA levels detected (Reynolds and Reynolds, 2011). There is evidence that lesions of the hippocampus produce significant deficits in spatial memory and working memory (Deacon et al., 2002). Recently, the role of the ventral hippocampus has gradually been revealed by studies of emotions, especially fear conditioning. Schizophrenics express diminished ability to recognize fearful faces and have disturbed fear perception (Holt et al., 2005). Animals whose ventral hippocampus was damaged by lesions reflected reduced fear responses (Kjelstrup et al., 2002). Furthermore, significant deficits of NAA in the ventral hippocampus may suggest that the PCP model reflects abnormalities of negative symptoms in

schizophrenia. Also, it is essential to mention that damage to this part of hippocampus has profound effects on the functioning of the frontal cortex shown as impaired working memory (Lipska et al., 2002). These rats also presented elevated locomotor activity, which may be comparable to the catatonia seen as a symptom in schizophrenia. This lesion study indicated that only neonatal damage may compromise cognitive abilities. Therefore, this suggests that the ventral hippocampus may be one of the first pathologies in the sub-chronic PCP model brain. This should involve more research to determine if the neonatal PCP model indicates the earliest ventral hippocampus damage.

It is important to notice that our concentration of NAA in the dorsal hippocampus was the lowest among groups, particularly when comparing to ventral hippocampus. NMDA receptors expression differs in these two regions (Pandis et al., 2006). Other study indicated that GABAergic neurones ratio compared to glutamatergic is higher in ventral than dorsal hippocampus in the mouse brain (Jinno and Kosaka, 2010).

All these arguments suggest that PCP may act differently on these two structures of the hippocampus. This division of the hippocampus had not been applied before, so further research could establish an overall strong link between abnormalities and NAA levels detected in humans compared to the PCP-induced damage. However, in our study we focused on the regions of the hippocampus together (i.e. DG and CA regions) and in this case NAA may not be sensitive enough to show differences between the regions of the hippocampus. There have been other noted neuronal deficits seen in the hippocampus in the PCP model, (Jenkins et al., 2010; Z. Abdul-

Monim, 2003) reported a reduction of parvalbumin containing interneurons in the rat hippocampus following PCP linking, as mentioned before, to cognitive deficits.

2.4.5 Investigating effects of sub-chronic PCP on NAA levels - Temporal Cortex

We found no significant (P= 0.161) changes in NAA in PCP treated animals when compared to vehicle. The role of this region has been emphasised since Kraepelin, who believed that the origin of auditory hallucinations were located in the temporal cortex (Krapelin, 1913). Further, Southard indicated that post-mortem examinations of brains from patients with schizophrenia indicated asymmetrical abnormalities in this region, specifically on left side (Southard, 1915). Finally, auditory hallucinations have been associated with temporal cortex abnormalities by neuroimaging studies (Barta et al., 1990; Buchsbaum, 1990; Ishii et al., 2000). NAA levels were shown to be significantly lowered in human post-mortem studies (Nudmamud et al., 2003a). Also, significance in this region was indicated from using the chronic PCP model (Reynolds et al., 2005) as well as rats housed in isolation (Harte et al., 2004). The discrepancy of our results could be due to different definition of the boundaries of the temporal cortex. Parameters of bregma points vary and dissected tissue were obtained from considerably smaller areas than these examined in isolated rats. Also, analysis of both sides of the temporal cortex could contribute to negative findings. There is extensive and consistent evidence that the asymmetry is present in this region in humans (Bilder et al., 1999; Galinska et al., 2007). Our study could have been improved by the division of the temporal cortex for the left and right site. This could allow differentiation and check if the asymmetry could be also seen in the PCP

model. Therefore, this research should be extended to evaluate the PCP effect on the asymmetry of the temporal cortex.

2.4.6 Conclusions

Sub-chronic PCP administration causes a reduction in NAA levels in the frontal cortex and ventral hippocampus. These suggest neuronal loss/dysfunction in these regions in PCP treated animals. In support of this finding we also found a deficit in cell density (as assessed by cresyl violet staining) in the frontal cortex. The work in this chapter with olanzapine, as mentioned before, shows the limitations in current treatments for schizophrenia that are available at this moment in time. The data presented demonstrates the use of NAA to investigate potential novel drugs as they come onto the market to assess their ability to target the underlying pathology associated with the subset of symptoms or the illness. However, the main limitation in the methods used in this study is that the work is carried out in post-mortem tissue which can only be accessed after sacrificing the animals, only allowing a single time point for the analysis. The use of MRS for assessing NAA levels *in vivo* will allow for complimentary studies to assess the neuronal dysfunction at various time points and also allow for assessment of the effect of treatment in the same animals. As such, the next studies will focus on determining NAA levels in the sub-chronic PCP treated animals *in vivo* using MRS.

Chapter 3

Investigating N-acetylaspartate levels in a preclinical model (NMDA receptor antagonist model) of relevance to schizophrenia – An *in vivo* Magnetic Resonance Spectroscopy Study

3.1 Introduction

The main interest in NAA was caused by the realisation that it represents the largest peak on proton magnetic resonance spectroscopy (Tsai and Coyle, 1995). It was first noted to be the most prominent peak in MRS with the work by (Barany et al., 1987; Fan et al., 1986; Luyten and den Hollander, 1986) as well as the connection to the fatal hereditary genetic disorder Canavan's disease. It has been seen that a perikaryal specific excitotoxin lesion of the rat striatum substantially reduced NAA levels at the lesion site (Guimaraes et al., 1995). Guimaraes reports the reduction in the NAA was mirrored with a comparable reduction in the NAA signal as measured by MRS, therefore validating the hypothesis that NAA measured by MRS was a reasonable surrogate for neuronal integrity. MRS was also one of the methods used to confirm reports of the neuronal localization (cerebellar granule neurons) of NAA, (Urenjak et al., 1992), (Urenjak et al., 1993). Although the work done revealed NAA's presence in the 0-2A progenitor and immature oligodendrocyte, the metabolite was not detected in mature oligodendrocytes. It is also because of the non-invasive ability of MRS as a diagnostic marker, when looking at neuronal loss or dysfunction (Burri et al., 1990), (Graham et al., 1994), (Pouwels and Frahm, 1998), (Leary et al., 1999), that it has become more valuable, as well as the ability for it to monitor disease progression. In MRS spectrum of normal human brain the major peaks observed from left to right on the spectrogram include myoinositol, choline, total creatine and NAA (Including NAAG), see figure 16. NAA representing the largest peak, in healthy brain tissue.

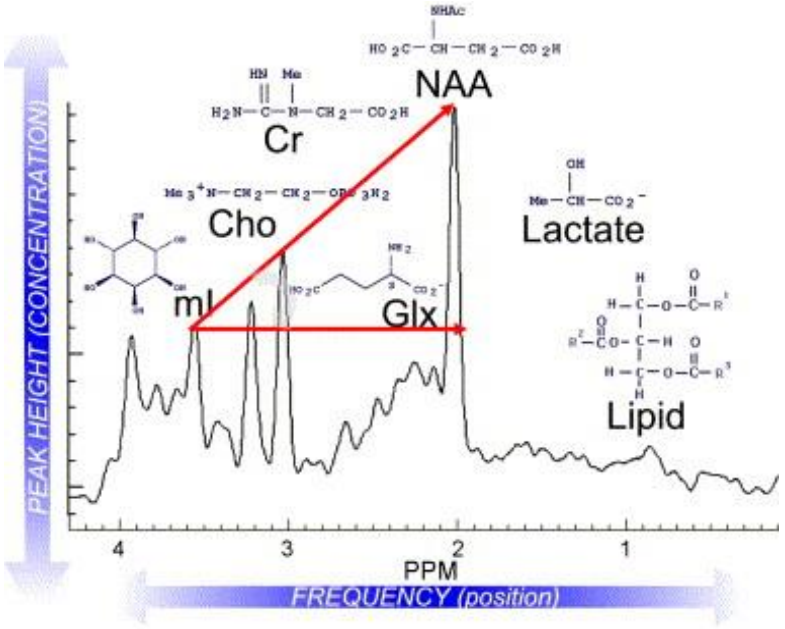

Figure 16: MRS Spectrum

Representative proton MRS spectrum of normal human brain with major peaks of interest depicted. Lactate and lipid signals are absent from this spectrum of a healthy individual. Hunter's angle (HA) refers to the approximate 458 angle formed by the peaks myo-inositol (mI), creatine (Cr), choline (Cho), and NAA, when they are present in normal proportions (NAA/Cr 1.5, Cho/Cr 0.75; mI/Cr 0.5) using short-echo-stimulated echo acquisition mode (STEAM) spectroscopy. Changes in HA can be applied to such common MRS diagnoses as tumour (HA 508), stroke, Alzheimer disease (HA 158), neonatal hypoxia (HA 458), or AIDS-related progressive multifocal leukomalacia (HA 08). Glx, glutamine and glutamate. Reprinted with permission, NeuroRx (Lin et al., 2005)

The acetate moiety of NAA is coupled through the amine nitrogen of aspartate and the three equivalent hydrogen atoms of the acetate group resonate in MRS with a single, sharp peak having a chemical shift of 2.02 ppm relative to the standard tetramethylsilane. While the peak at 2.02 is prominently attributable to NAA, this signal includes smaller contributions from other acetylated compounds such as from the neuron-specific dipeptide, N-Acetylaspartylglutamate (NAAG) (Caramanos et

al., 2005), N-acetylneuraminic acid (Varho et al., 1999), and underlying coupled resonances with glutamate and glutamine. It is therefore important to factor in that NAAG will contribute about 15 - 25% of the acetate signal that is usually ascribed to NAA (Barker et al., 2006; Pouwels and Frahm, 1997, 1998). This means that the reductions in the NAA peak associated with various brain disorders either involve coincident reductions in NAAG, or they may underestimate the drop in NAA in situations where NAAG levels remain constant.

It has been noted that MRS has been used in the clinic to map the decreased NAA levels in a number of neurological disorders, with the exception of Canavan's disease which shows an increase in the levels of NAA (Wittsack et al., 1996). Diseases and disorders that have shown decreases in NAA include, Stroke (Lai et al., 1995), Alzheimer's disease (Klunk et al., 1992), epilepsy (Matthews et al., 1990), brain cancer (Gill et al., 1990), multiple sclerosis (Davie et al., 1994) AIDS dementia disorder (Menon et al., 1992), depression, bipolar disorder, psychiatric disorders and schizophrenia.

It is important to note that deficits in NAA have also been shown to be reversible following an acute brain injury (De Stefano et al., 1995), (Kalra et al., 1998). Taken together, the evidence may be another indicator that NAA is a marker of both neuronal loss and cellular dysfunction.

It has previously been reported that rats can show NAA levels effectively through scanning with MRS (Bustillo et al., 2004) and that there is no effect of antipsychotics on NAA levels when compared to vehicle treated animals. These results were also mirrored in a study where long term treatment with haloperidol, showed no effect on NAA levels when compared to vehicle treated controls (Bustillo et al., 2006). One note of caution in such studies are that the effects of chronic antipsychotic treatment are carried out in rats with normal brain chemistry, equivalent to looking at the effects of antipsychotics on a healthy and not diseased brain.

It is important to understand some of the limitations of MRS and therefore the leaning of some research groups to other analytical methods of measuring NAA. Most studies of NAA using MRS use small volumes of interest (VOIs). The VOIs must be placed away from the skull to avoid contamination from subcutaneous lipid and bone marrow signals, therefore missing most of the cortex (Inglese et al., 2004). This uses the assumption that metabolic abnormalities only occur in the specific VOI. Therefore in most CNS disorders where the effects are largely diffuse, these processes will miss 80-99% of the brain. This renders the true extent of their load susceptible to extrapolation errors (De Stefano et al., 2002).

It is important to understand the limitation of the reference compounds currently used in the literature (Barker et al., 2006). The most common reference molecule used in analysis of NAA in brain samples is Creatine (Cr) and is used in ratios of NAA/Cr. The advantage of Cr being used as a reference is that it is an internal

reference from the same voxel as the NAA being analysed, therefore cutting the time for analysis. However, Cr levels are known not to be a constant in a brain region and therefore can skew the NAA/Cr ratio showing higher or lower than normal levels of NAA giving a false positive or negative (Hetherington HP, 1994; Soher BJ, 1996; Stockler S, 1994). Another reference used in increasing amounts is water referencing. Water referencing has previously been shown to be a reliable single voxel molecule (Soher et al., 1996) however, there are some situations where water referencing is not the most reliable; due to water changes in the brain with neonatal studies or with some MR imaging where the water reference may not be available. It has been accepted in the research group at the University of Manchester that in some cases with a high power magnet, with a good resolution, that a reference molecule is not needed. This removes any issues that may occur causing the NAA data to skew providing an absolute level of NAA between the groups. Although it prevents a comparative quantification of the NAA to other papers, it does allow a highly accurate comparison between groups to be made.

3.1.1 Aims

As demonstrated in chapter 2, looking at the effect of chronic treatment with novel compounds in the brains from PCP treated animals, may provide a platform for testing novel treatment strategies with the ability to identify compounds that target the underlying pathology, as assessed through the translation biomarker NAA. Utilising MRS to look at NAA in the same animal over time would provide a powerful tool for such an approach.

The main aims of the chapter were to:

- Demonstrate that NAA can be clearly and accurately measured using MRS in a preclinical model of schizophrenia.

- Validate the *ex vivo* findings and to assess NAA in the sub-chronic PCP model using in vivo MRS.

- Determine if cognitive deficits were observed alongside the NAA changes.

- Associate the *in vivo* MRS findings with *ex vivo* HPLC analysis of post-mortem tissue from the same animals.

3.2 MRS Materials and Methods

3.2.1 Animals

This study used 10 adult female (230-280 g) hooded-Lister rats (Charles River or Harlan, UK). All 10 rats underwent testing in the novel object recognition task (NOR), MRS scanning and HPLC determination of NAA. Rats were housed (5 per cage) in two-tiered solid floored plastic cages by tecniplast (462 x 403 x 404 mm) containing sawdust, paper sizzle nest (Datesand, Ltd., Manchester, England) and fun tunnels (plastic environmental enrichment tubes., Datesand, Ltd., Manchester, England). Food (Special Diet Services Ltd., Essex, England) and drinking water was available *ad-libitum* in the home cage. The rats were disturbed only for weighing and cleaning, which consisted of changing the cage once per week. Rats were housed in a single sex colony which was maintained under a constant temperature of approximately 21 ± 1 °C and humidity (40-50 %) under a 12 h light:dark cycle (lights on at 0700 h). All experiments were conducted during the light phase and were carried out between 0900 h and 1630 h. All studies were compliant with the Animal Scientific Procedures act (1986) and approved by the University of Manchester Ethical Review Process.

3.2.2 Experimental procedure timeline

Prior to the experiment start, a test animal was scanned to locate the frontal cortex and refine the protocol for scanning the brain on the MRS scanner. Voxel size had

to be chosen to provide the best resolution whilst not causing any other brain anatomical structures leaking into the target scan area. After voxel size had been set the resolution and scanning procedure was set and refined then programmed into the system to reduce scanning setup time. Before the PCP dosing regimen all 10 rats were scanned in the MRS scanner over a two day period. The rats were then dosed with either Vehicle or PCP and left for 6 weeks after the final day of washout. On the second scanning session all 10 rats, 5 vehicles and 5 PCP, were rescanned again over a two day period. After the end of the experiment rats were sacrificed using a schedule 1 technique and the brains were taken for HPLC analysis.

Figure 17: MRS Experimental timeline

3.2.3 Sub Chronic PCP dosing regimen

PCP dosing was carried out as described in chapter 2.10. Rats were separated into two cohorts, 5 vehicle (0.9% saline solution) and 5 PCP (2 mg/kg, bi-daily for 7 days).

3.2.4 Anaesthetic Procedure

Rats were removed from their home cage and placed into the anaesthetic chamber. Rats were anesthetised with 4% isoflurane in an O_2 mix at a flow rate of 1.5 L/min.

Rats were then transferred to the scanner and placed in a stereotaxic frame, held in place with a tooth hook and ear bars. The frame was placed in the MRS scanner and positioned so that the area of interest was located at the isocenter of the magnet. During scanning anaesthesia was maintained at 2%. Respiration and temperature was monitored at all times throughout the experiment. Temperature was maintained with constant flow of warm water around the scanning bed at 37.5 – 38.5ºC. Temperature and respiratory monitors can be seen in figure 18.

3.2.5 Data acquisition and scanning equipment

NAA amplitudes were acquired using an AGLIENT 7 Tesla magnet, shown in figure 18, 16 cm core at the University of Manchester imaging suite. The scanning was carried out using Paravision 5 to provide the scan and analysis. Rats were first scanned and checked visually for any scanner artefacts and anatomical anomalies then a 5 mm^3 voxel placed in the area of interest for shimming and line width. Voxel size used for acquisition was placed centrally to the previous area and was 6 x 2 x 2 mm. Shimming and line width was re-checked for resolution before proceeding onto water suppression. Evolution time for all scans were within the range of 28 – 32 ms with a line width range of 14.9 – 12.7 Hz. If the evolution time or the line width did not meet desired tolerances then the target voxel was repositioned to acquire a better resolution. The brain area was scanned for 128 scans to provide a low resolution check in case of any abnormalities in the spectrum. If the spectrum showed no abnormalities or artefacts the scan was increased to 512 and repeated. Final scans took 20 minutes to complete, with the whole process requiring 45 - 60 minutes from when the rat was placed into the anaesthetic chamber to returning to its home cage to

recover. Post processing was carried out using jMRUI v5.1 written by Merlijn Janssen and Danielle Graveron-Demilly. Water peaks were removed as were any lipid infiltration into the spectrum before the spectrum were analysed. NAA MRS spectrum peaks as shown in Figures 20 & 21 show the targeting of the NAA peaks in jMRUI. Following post processing the NAA peak must be identified as found between 2.0 and 8.0 ppm. The most prominent resonance of NAA is found at 2.01 ppm, from the CH_3 group of the N-acetyl part of NAA. Also three doublet-of-doublets centred at 2.49, 2.67 and 4.38 ppm corresponding to the protons of the aspartate CH_2 and CH groups. A broad doublet is also prominent at 7.82 ppm from the amide NH proton, however, this peak is temperature dependant (Govindaraju et al., 2000). The quest peak for NAA must be focused over the NAA peak to cover the largest amount of NAA peak possible by adjustment of the pink NAA spectrum left or right.

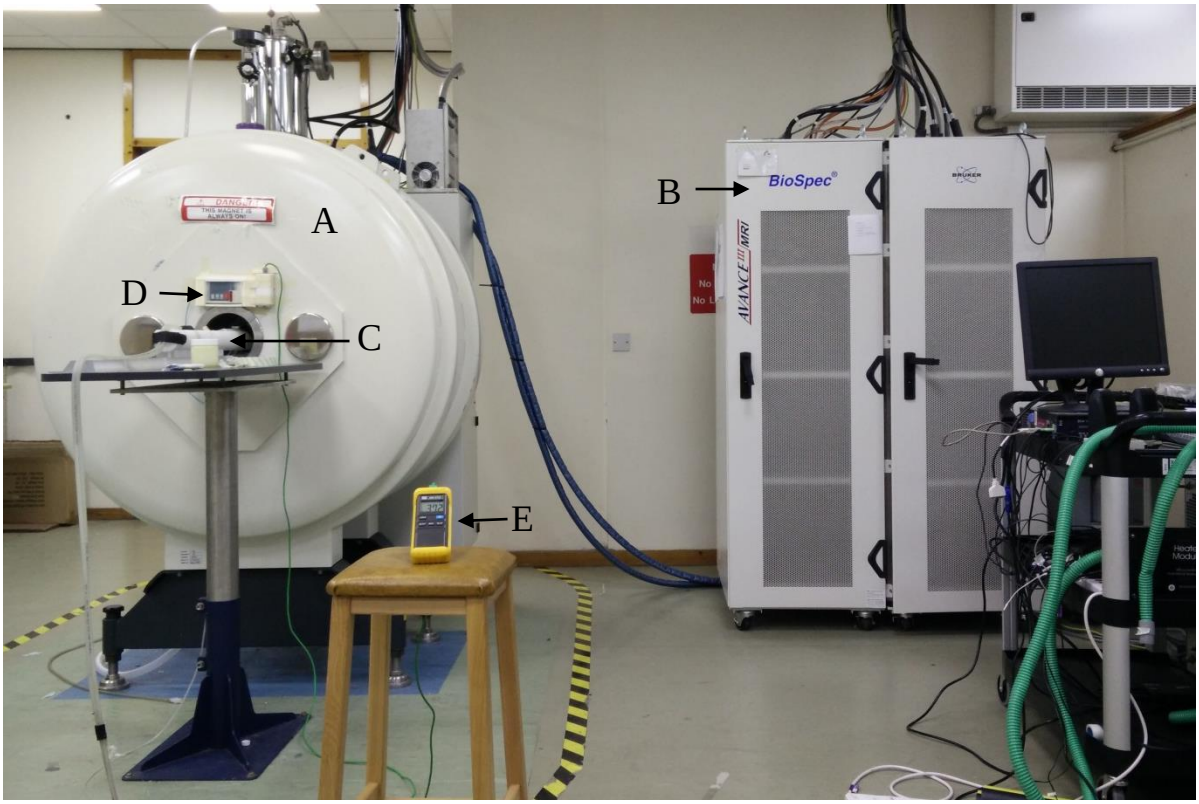

Figure 18: Photo of the MRS 7T scanner.

Shown in the image is the 7 T magnet (A), gradient controls (B), rat scanner bed (C), respiratory monitor (D) and temperature sensor (E).

3.2.6 Novel Object Recognition Experimental Protocol Apparatus

NOR experimental procedure adapted and taken from the work by Dr Ben Grayson's thesis (Grayson, 2012a; Grayson et al., 2014; Grayson et al., 2007; Grayson et al., 2015; Neill et al., 2010). The testing apparatus consisted of an open box made of Perspex (52 cm L; 52 cm W; 31 cm H), with the sides painted black and the floor white and divided into 9 separate squares (17.3 cm x 17.3 cm) for the purpose of line crossings shown in figure 19. NOR boxes were placed 27 cm above the floor on a static stand. The objects were made of glass (250 ml drug bottle filled with water)

and metal (Coke can, unopened). The height of the objects was approximately equal (10 cm ± 2 cm) and they were heavy enough not to be displaced by the animals. Objects were positioned 6 cm away from the walls of the box, in opposite corners. Prior to each trial, ethanol (10 % v/v) was used to clean the objects in an attempt to remove any olfactory trails on the objects and in the box.

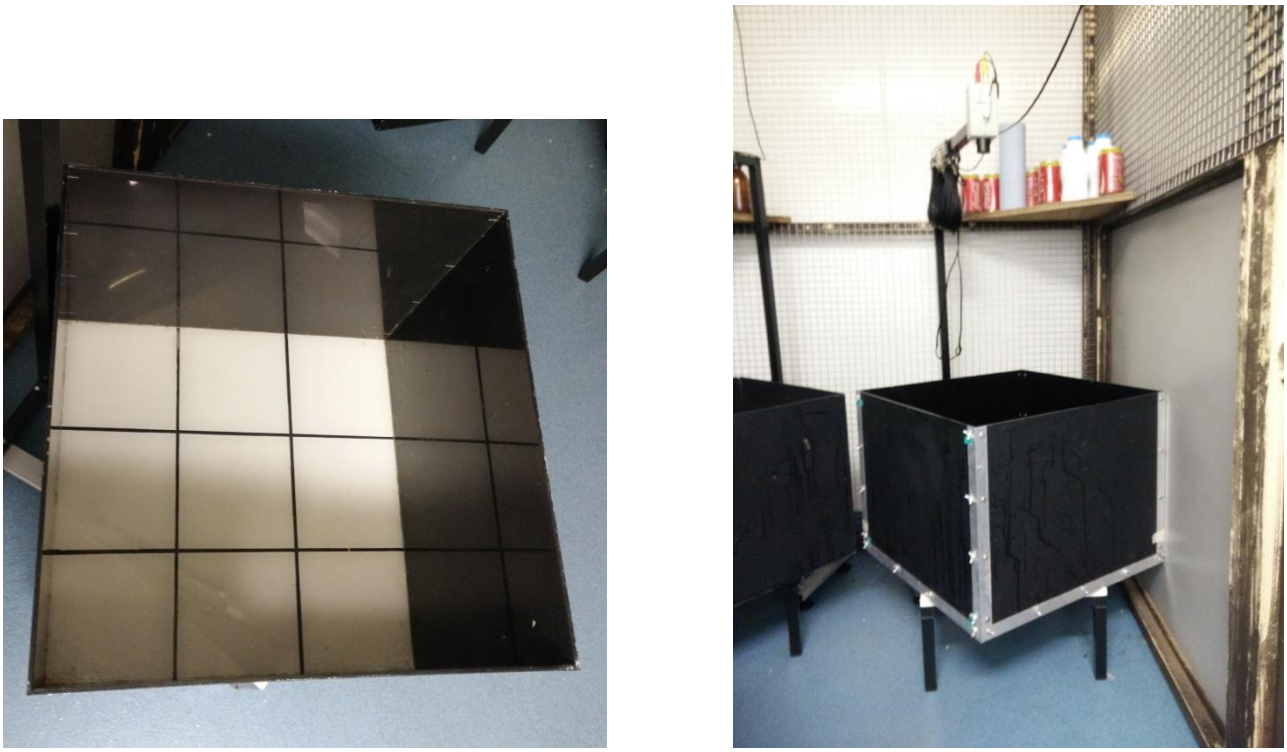

Figure 19: Showing the NOR testing boxes.

Image on the right shows the line crossing markings and the inside of the testing chamber. Image on the left shows how the box is elevated above the floor and the

positioning of the recording equipment. Objects used for NOR testing can be seen behind the testing apparatus.

3.2.7 Testing procedures

Rats were habituated to the empty NOR apparatus and test room environment in cage groups of 5 for 3 consecutive days for 10 min, allowing one of the habituation days to precede the test day. In the acquisition trial the rats were exposed to two identical objects, either the coke can or drug bottle, for a period of 3 min. The rats were returned to a holding cage (small perspex box with a sawdust covering) for a 1 min ITI. During this period, both objects were removed, the entire box and objects were cleaned with an ethanol solution (10 % v/v) to remove any lingering olfactory trails. A novel object and a triplicate copy of the familiar object was replaced into the NOR test box. Following the 1 min ITI, rats were returned to the NOR apparatus to explore the familiar and novel objects for a period of 3 min. The location of the novel object in the retention trial was randomly assigned for each rat using a pseudorandom schedule.

3.2.8 Scoring protocol

Object exploration was defined as the rats sniffing, licking or touching the objects with forepaws whilst sniffing. Leaning against, turning around, standing or sitting on the objects were not considered object exploration (Grayson et al, 2007). The

exploration time (s) of each object in each trial was recorded manually using two stopwatches by an experimenter blind to treatment.

If an animal failed to explore one or both of the objects (for less than 1 (s) in the acquisition or retention trial it was excluded from the final data analysis.

Discrimination Index (DI) as used in NOR experimental data allows for statistical cross comparison between the Vehicle and PCP animals, which would not normally be possible (Ennaceur and Delacour, 1988). DI is calculated by the exploration time of the novel object (T_N), minus the exploration time of the familiar object (T_F) divided by the exploration time of the novel object added to the exploration time of the familiar object. $DI = (T_N - T_F) / (T_N + T_F)$. DI represents the difference in exploration time expressed as a proportion of the total exploration time of the two objects used in the retention trial.

Line crossings were recorded by counting the total number of line markings on the floor of the NOR box crossed by the rats during the acquisition and retention trials. One line crossing was counted when the base of the rat's tail crossed over a line.

3.2.9 Brain Dissection

At the end of the experiments animals were sacrificed by CO_2 inhalation. Brains were quickly removed and frontal cortex (anterior to bregma +3.20), was collected

(Paxinos and Watson, 1998) See figure 5). The analysis included bilateral hemispheric dissection. Dissected tissues were immediately frozen on dry ice and stored at -80 for further analysis.

Frozen sections were then weighed and 10 times their volume of 0.1 M perchloric acid was added. Tissues were homogenised to precipitate proteins and centrifuged for 5 min at 12,000 x g to form a pellet (Eppendorf Centrifuge 5415 C). The supernatant was removed and used to prepare the samples for analysis. Samples consisted of 50 µL of supernatant added to 920 µL of 50 mM phosphate buffer and 30µL sodium carbonate. Samples were extracted using anion exchange columns (Figure 5).

3.2.10 HPLC determination of NAA levels

Please refer to chapter 2 (2.2.5 - 2.2.8) for the full HPLC methodology.

3.2.11 HPLC Experimental Overview

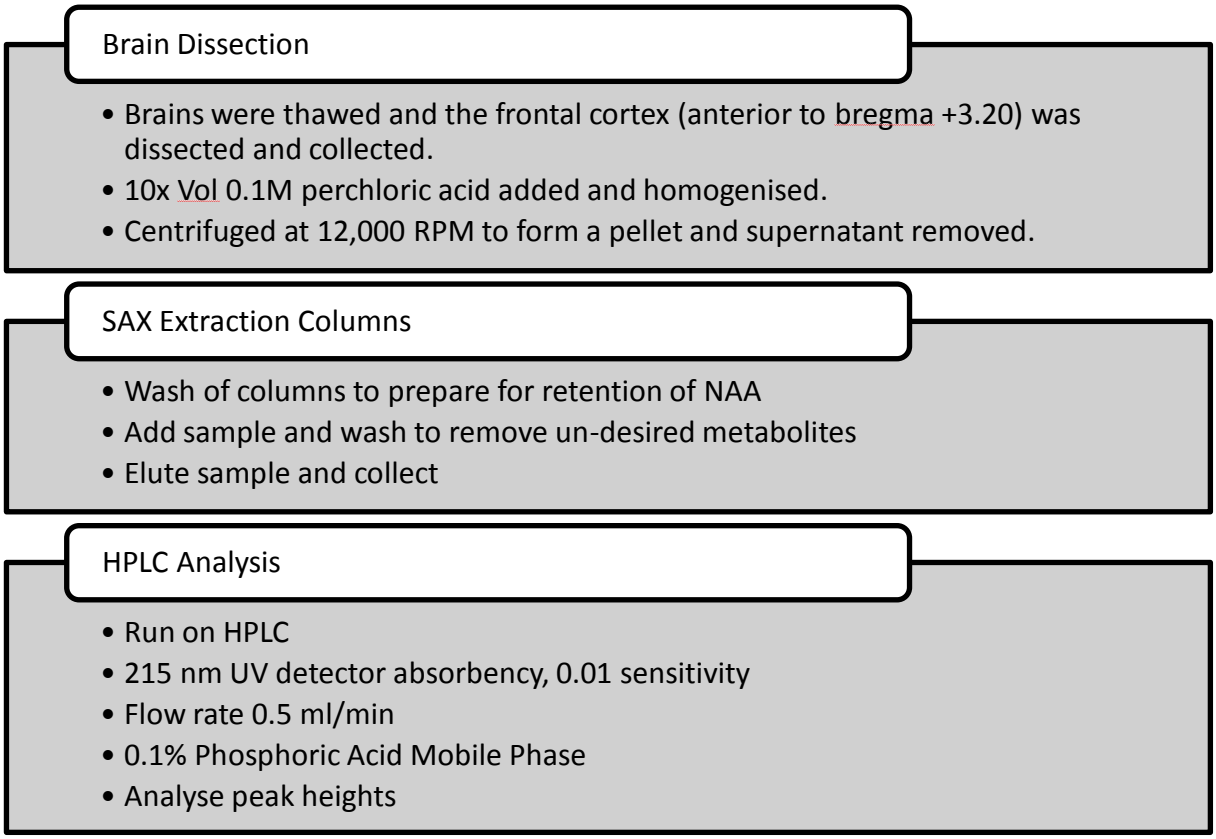

Table 8: MRS Experimental Overview

3.2.12 Statistical Analysis

Determination of deficits in NAA amplitudes in the frontal cortex in vehicle and PCP rats was evaluated by two-way ANOVA and t-test. Determination of deficits in the NOR experiment was evaluated by Student's t-test between object A & B, novel and familiar for vehicle or PCP. DI and line crossings were analysed by Student's t-test. Determination of deficits of NAA using HPLC were analysed by Student's t-test. The statistical analysis was completed on SPSS/PASW version 20. A p value < 0.05 was considered to be significant.

3.3 Results

3.3.1 MRS Results

Analysis of the data using a two way ANOVA presented that a cross comparison of both time points and two groups of vehicle and sub-chronic PCP treated rats showed a significant effect ($F_{1,\ 4}$=7.995, P<0.05; Figure 22).

Analysis of the data with a two way ANOVA showed that treatment with sub-chronic PCP presented a significant effect between the dose groups between the two testing points ($F_{1,\ 4}$=15.011, P<0.05; Figure 22). Following a post-hoc Student's t-test comparison between the drug groups on separate days showed there was no significant difference between the dose groups on week 0. However, there was a significant (P<0.05) reduction in the levels of NAA in the sub-chronic PCP rats at 6 weeks when compared to vehicle treated rats.

Analysis of the data using a two way ANOVA presented no significant difference between the two groups when comparing the two time points ($F_{1,\ 4}$=0.274, NS; Figure 22). Following a post-hoc Student's t-test comparison between the two time points showed no significance in the vehicle rats when compared between week 0 and week 6. However, there was a significant (P<0.05) reduction in the levels of NAA in the sub chronic PCP rats between the two testing times.

	NAA Baseline Amp	NAA Baseline SD	NAA 6 Weeks	NAA 6 Weeks SD
V	3675.0	1025.0	3516.0	1087.0
V	4210.0	908.4	7400.0	787.8
V	5025.0	499.9	5989.0	792.8
V	4865.0	465.7	6526.0	1762.0
V	6079.0	1884.0	4084.0	931.0
Adv	4770.80	956.61	5503.00	1072.10
SEM	406.21	256.57	736.31	180.97
P	3445.0	903.4	3366.0	460.3
P	4717.0	1426.0	3502.0	982.4
P	5668.0	2083.0	3940.0	1806.0
P	4463.0	1218.0	3815.0	995.2
P	5276.0	2108.0	2034.0	1269.0
Adv	4713.80	1547.68	3331.40	1102.58
SEM	380.74	238.66	340.43	219.21

Table 9: Raw data as collected from jMRUI, Quest output. Standard deviation is derived from multiple scans in the scanning technique (512 scanning passes).

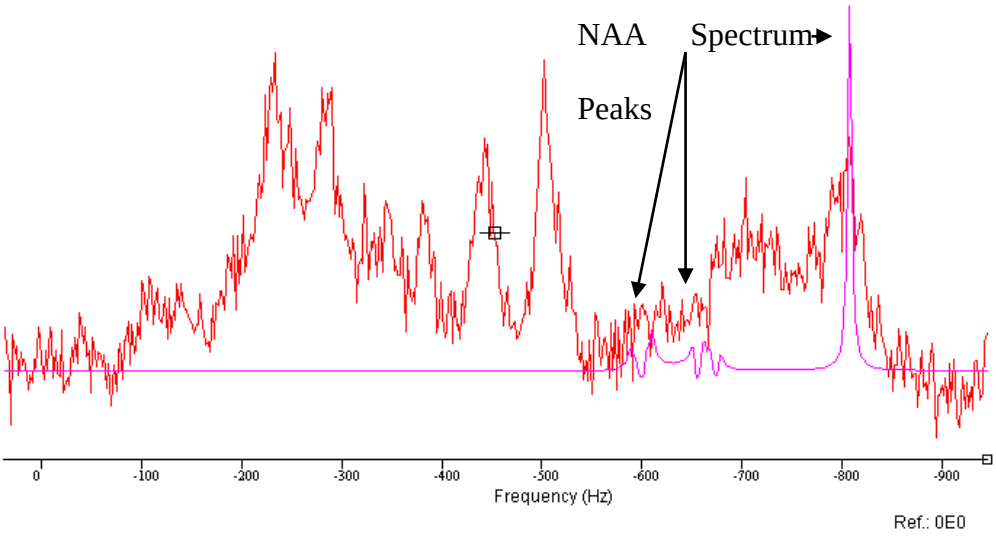

Figure 20: Example experimental MRS Spectrum; Vehicle

Example of spectrum showing Vehicle animal Cage 2 Rat 3 analysed in jMRUI. Red line is the spectrum as shown after pre-processing with the water and lipid infiltration removed. Pink line is the NAA spectrum guide to show location of NAA amplitude in the spectrum. Peak shown is amplitude 5989.

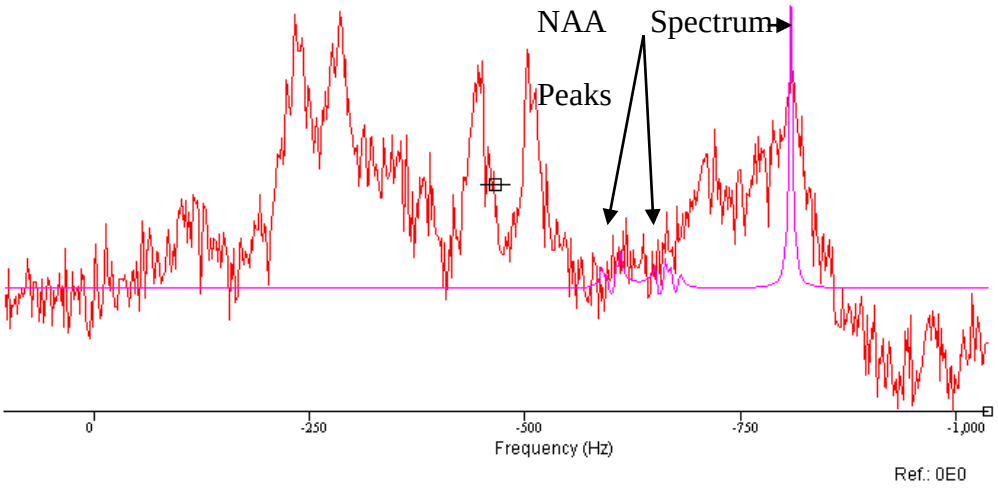

Figure 21: Example experimental MRS Spectrum; PCP

Example of spectrum showing PCP animal Cage 3 Rat 2 analysed in jMRUI. Red line is the spectrum as shown after pre-processing with the water and lipid infiltration removed. Pink line is the NAA spectrum guide to show location of NAA amplitude in the spectrum. Peak shown is amplitude 3502.

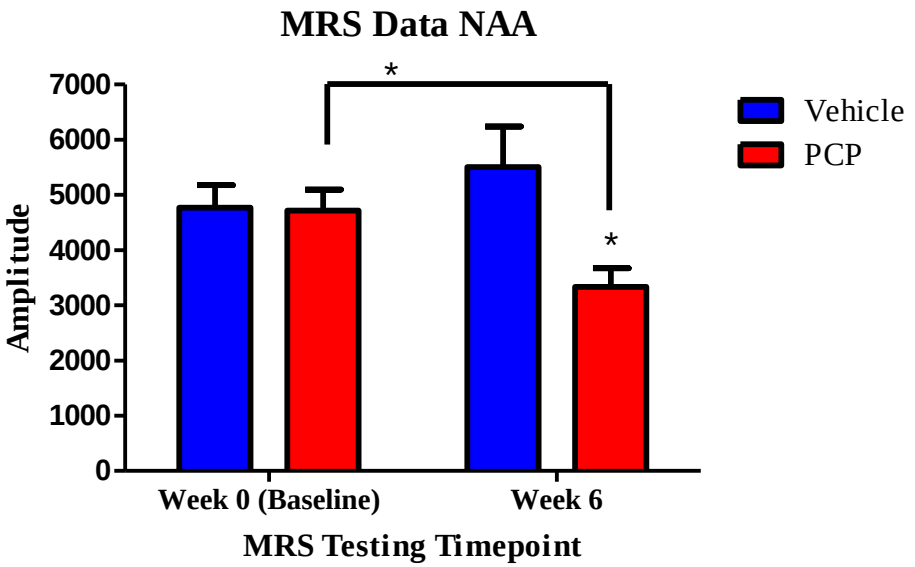

Figure 22: The effect of sub-chronic PCP treatment (2 mg/kg, i.p. for 7 days followed by 7 day washout period) or saline (vehicle, i.p.) on the amplitude of NAA in a MRS spectrum separated by 6 weeks. Data are shown as the mean ± S.E.M. (n = 5 per group). *P<0.05; significant reduction in amplitude in the PCP group at week 6 when compared to week 0. *P<0.05; significant reduction in the amplitude in the PCP group at week 6 when compared to the vehicle group, students t-test.

3.3.2 NOR Results

Analysis of the data with a Student's t-test showed that treatment with sub-chronic PCP did not produce any significant effect in the acquisition phase of the NOR experiment (Vehicle; $t(4)=-0.715$, PCP; $t(4)=0.346$, NS; figure 23). Rats from both treatment groups presented an equal amount of exploration on both objects.

Analysis of the data with a Student's t-test in the retention phase shows that treatment with sub-chronic PCP produced no significant effect in the retention phase of the NOR experiment ($t(4)=-0.238$, NS; figure 24). Whereas the vehicle rats significantly, ($t(4)=-5.207 P<0.01$) explored the novel object compared to the familiar object following a 1 min ITI being placed back into its holding cage. This difference was not seen in the group of animals treated with PCP. These rats spent a similar amount of time exploring both objects in the retention phase.

DI data showed a significant ($P<0.05$, figure 25) effect of the PCP when compared with vehicle following Students t-test on the rats ability to discern between novel and familiar objects in the retention phase following 1 min ITI.

Line crossings showed no significant (Figure 26) effect of the PCP when compared with vehicle following Students t-test in the acquisition and retention trial in the NOR test.

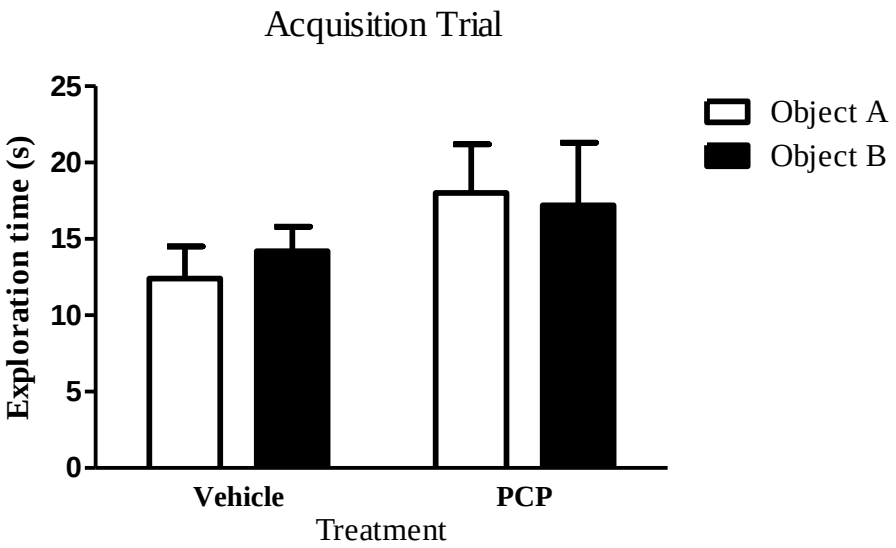

Figure 23: The effect of sub-chronic PCP treatment (2 mg/kg, i.p. for 7 days followed by 7 day washout period) or saline (vehicle, i.p.) on exploration times (s) of identical objects in the 3 min acquisition trial in the NOR test in female rats. Data are shown as the mean ± S.E.M. (n = 5 per group).

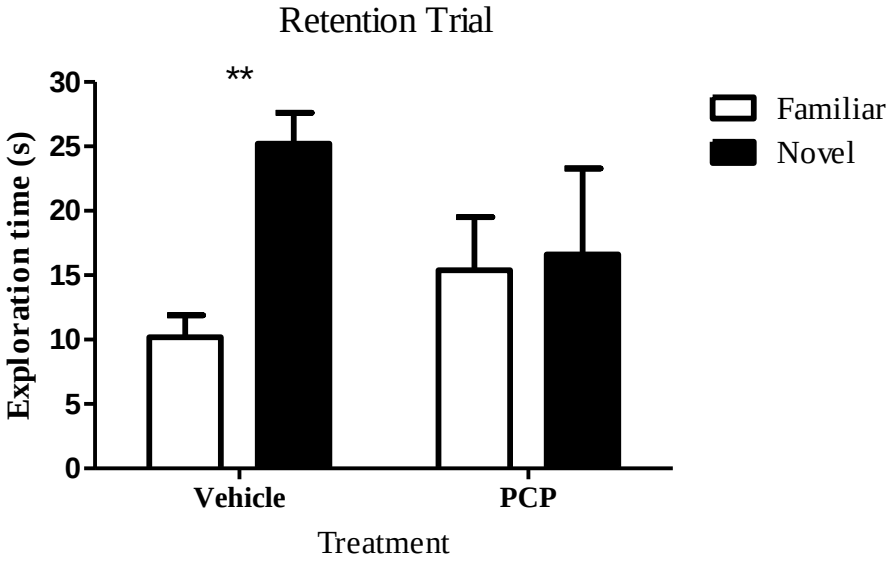

Figure 24: The effect of sub-chronic PCP treatment (2 mg/kg, i.p. for 7 days followed by 7 day washout period) or saline (vehicle, i.p.) on exploration time of a familiar object and a novel object in the 3 min retention trial in the NOR test in female rats following 1 min ITI in the holding cage. Data are shown as the mean ± S.E.M. (n = 5 per group). **P<0.01; significant difference between the time spent exploring the familiar compared with the novel object, Students t-test.

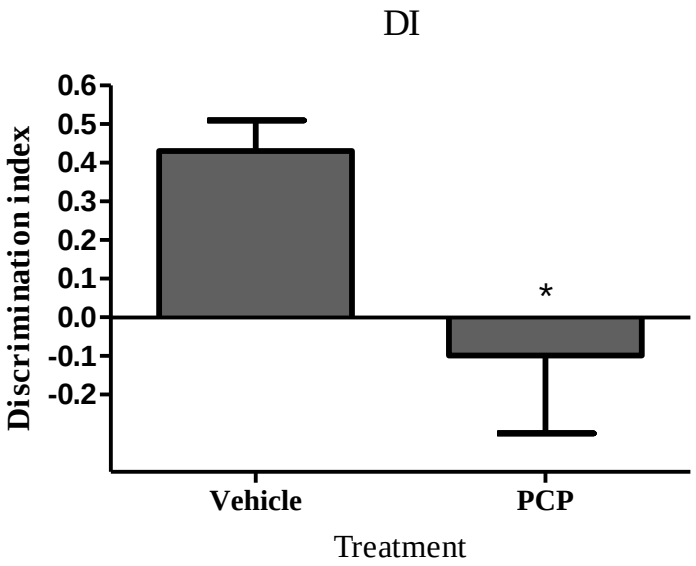

Figure 25: The effect of sub-chronic PCP treatment (2 mg/kg, i.p. for 7 days followed by 7 day washout period) or saline (vehicle, i.p.) on the discrimination index in the NOR test in female rats following 1 min ITI in the holding cage. Data are shown as the mean ± S.E.M. (n = 5 per group). *P<0.05; significant reduction in DI when compared to vehicle, Student's t-test.

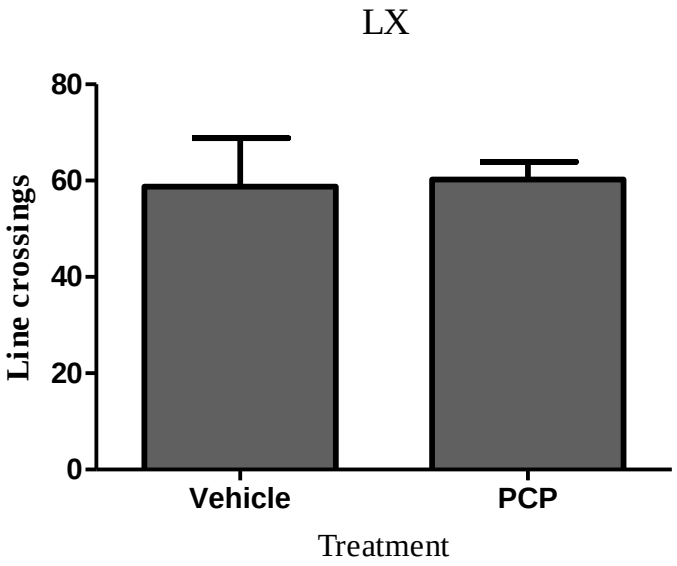

Figure 26: The effect of sub-chronic PCP treatment (2 mg/kg, i.p. for 7 days followed by 7 day washout period) or saline (vehicle, i.p.) on the total number of line crossings in the acquisition and retention trial in the NOR test in female rats following a 1 min ITI in the holding cage. Data are shown as the mean ± S.E.M. (n = 5 per group). No significant reductions following Student's t-test.

3.3.3 HPLC Results

Analysis of the data using a Student's t-test presented a significant (P<0.001, Figure 27) reduction in the levels of NAA in the PCP group when compared to the vehicle group.

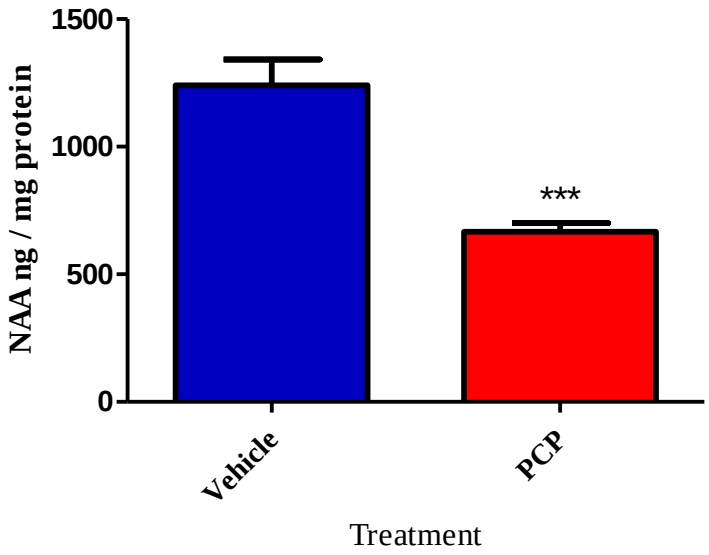

Figure 27: The effect of sub-chronic PCP treatment (2 mg/kg, i.p. for 7 days followed by 7 day washout period) or saline (vehicle, i.p.) on the levels of NAA in a HPLC determination of NAA. Data are shown as the mean ± S.E.M. (n = 5 per group). ***P<0.001; significant reduction in levels of NAA in the PCP group when compared to vehicle, Student's t-test

3.4 Discussion

In this chapter we found, following a sub-chronic PCP dosing regimen, a reduction in the levels of NAA in the rats that were scanned, 6 week interval post PCP washout period, compared with baseline. These data were supported by the NOR behavioural test which showed a deficit in the cognitive behaviour as an inability of the PCP rats to discriminate between the novel and familiar objects in the retention phase. The support for the PCP model being used for a model of cognitive deficits has been shown multiple times as can be seen by the work from our lab (Neill et al., 2010). It is PCP's ability as an NMDA receptor antagonist to cause schizophrenic like effects in a brain after a chronic dose over 7 days. It is believed that the NMDA receptor hypofunction, caused by the chronic dosing regimen, leads to a glutamatergic dysfunctioning and resulting pathology that associates with aspects of what is reported in schizophrenia. Disruptions in normal neuronal functioning, or dysfunction, have been shown in previous chapters in this thesis to cause a reduction of the levels of NAA in specific brain regions. This supports the idea that NAA can be used as a neuronal, axonal number or viability marker and that a reduction in NAA can suggest either a neurodegenerative or neurodevelopmental process in the FC in schizophrenia.

In this present experiment we decided to use an absolute value of NAA rather than the more common NAA ratios that are published in a large amount of research papers available. With an accurate spectrum, gained from the use of a high power MRS scanner, there is no need for a ratio as it has been noted that the NAA/Cr, NAA/Co ratios typically seen can sometimes show significant increases or decrease

in NAA only due to the fact that it is the Cr/Co ratio changing (Yamasue et al., 2002). It has been reported, for example, that the choline levels in schizophrenic patients' brains both increase (Block et al., 2000; Buckley et al., 1994) and decrease (Bartha et al., 1997; Bertolino et al., 1996; Deicken et al., 1997b; Ende et al., 2000) in the frontal lobes in comparison to controls. This could cause false positives/negatives in the data presented as changes could be due to Cr or Co changing and not to an increase or decrease in NAA levels. Due to this we chose to display NAA as amplitude to prevent any ratio bias in our data. We believe that, since reductions were seen in both the MRS analysis and the HPLC analysis to similar effect (~35-40% reduction in both MRS and HPLC), this method of data processing is an adequate representation of what is happening to the NAA *in vivo*.

In the current chapter our results show there is a significant ($P<0.05$) decrease in the levels of NAA, 6 weeks post sub-chronic PCP treatment, when compared to vehicle animals. Significance ($P<0.05$) was also found in the PCP group when compared to its baseline control scan. These data show that there is a marked neuronal dysfunction observable following PCP dosing that can be measured with NAA in an *in vivo* model of schizophrenia.

Work by (Bustillo et al., 2012) used a chronic PCP model of schizophrenia by dosing 14 injections (2.58 mg/kg/injection, i.p.) with male Sprague-Dawley rats. Injections were given daily for the initial 5 days, then at days 8, 10, 12, 15, 17, 19, 22, 24 and 26 using the methodology from (Reynolds et al., 2005). Rats were sacrificed three days after the last injection and then brain sections sliced and

prepared for scanning with a Bruker 11.7 T MRS scanner. This chronic model of schizophrenia showed a significant reduction in the levels of NAA in both the MFC and Nucleus Accumbens when compared to vehicle animals. This literature supports our findings in this chapter of the thesis, however, there are interesting differences in the methodologies used. Firstly the work done by Bustillo and colleagues (Bustillo et al., 2012) used a post mortem scanning model which differs from scanning *in vivo*. Even though both findings showed a reduction in NAA the work done in this thesis is more translational to the clinic as it was shown in live subjects. The PCP model has previously proved to be successful at mimicking the pathology of schizophrenia patients, and shows stable neuronal dysfunction at six weeks post PCP treatment. This is supported by the behavioural data presented alongside the MRS data in this chapter showing its ability to cause observable cognitive deficits

In a study by (Poland et al., 1999) they used a model of pre and post-natal stress by restriction of movement for one hour twice daily between days 14-21 of gestation in Sprague-Dawley rats in comparison to non-stressed rats. At 24-36 h after birth, pups were cross fostered to stressed or non-stressed mothers with only one pup from the same litter in a group. Pups were weaned at day 22 and then sacrificed at day 120. Brains were homogenised and scanned using a BRUKER 8.45 T MRS scanner. This model shows a significant reduction of NAA levels in both pre and post-natal animals when compared to controls in the left hemisphere. As much as the experimental protocols and model of pre and post natal stress differ from our PCP model and tissue scanning, it is important to show that the NAA marker has previously been successful in a variety of models, to good effect, to show changes in brain neurochemistry and neuronal functionality. Notably there is only a deficit

found in the left hemisphere of the brain and not in the right side. This is different from our finding as we scanned both the left and right sides of the FC together. This could suggest that in future studies of NAA in the FC using the PCP model should split both hemispheres and compare the data. However, if the size of the voxel is reduced you compromise the sensitivity of the scan as you cannot account for whole region changes.

An interesting comparison study by (Napolitano et al., 2014) looked at the NAA levels in the prefrontal cortex using MRS analysis of NAA in the acute ketamine and isolation rearing model. They found no change following ketamine treatment, in both the non-isolates and the isolates on NAA levels. In our PCP model, PCP has been shown to induce a clear pathological effect on the brain in sub-chronic treated animals. This is such a robust deficit that we can observe this deficit 6 weeks post treatment. In unpublished findings in our lab we have shown that this deficit can last over 12 months post treatment. This shows that the PCP causes a permanent dysfunction as can be shown by our found cognitive deficits in NOR. In comparison, the isolation rearing model shows no NAA reductions showing that there is no real robust pathological change. This, therefore, proves to us that the PCP model is the most robust to model schizophrenia.

In clinical comparison the work by (Shirayama et al., 2010) used 19 schizophrenia patients compared with control subjects with similar age and IQ. Schizophrenia patients met the DSM-IV criteria for schizophrenia and no other psychiatric disorders or drug dependence, being clinically stable for at least three months and all

but one receiving second generation neuroleptics. Subjects were scanned with a Signa Exite 3 T MRS scanner with voxel sixe 28 x 30 x 22 mm in the MPC. This work shows a significant reduction in the performance on prefrontal cortex related cognitive function tests in Verbal fluency (letter and category), WCST, Trail making test (A & B) and DSDT with distractor. Significant reduction was shown in MRS scan results of NAA / (glycerophosphorylcholine + phosphorylcholine) and Glutamine / Glutamate in the schizophrenic patients when compared to control subjects. These findings support data in this chapter and show the translation ability of the PCP model and NAA as a biomarker from pre-clinical analysis to the clinical studies of NAA.

A similar study also supports the findings in this chapter by (Pae et al., 2004) with analysis of 24 in-patient schizophrenics with an even male / female split and mean illness duration of 3.6 years in comparison to 20 control healthy subjects. Subjects scanned on a 1.5 T MRS system targeting the frontal lobe with a 2 x 2 x 2 cm voxel. A large decrease in the NAA/Cr ratio was shown when both left and right lobes were combined, when compared to normal controls. It is important to note that the patients in this study were currently taking antipsychotics. This clearly demonstrates that the compounds were not having an effect of the pathology as the NAA deficits are still present. This is a clear sign that there is a need for more targeted treatment strategies for schizophrenia. Although this group used a much lower resolution scanner than we used, their work supports the findings in this chapter as well as the translation ability of this model and NAA.

NOR is a measure of visual learning and memory, a cognitive domain, affected in patients with schizophrenia. We use this behavioural test to show a subjective behavioural result that can be compared to real world cognitive deficits. In this chapter we found no significant difference in the acquisition phase of the NOR experimentation for both Vehicle and sub-chronic PCP groups 63 days after dosing. These results are standard within the literature and to be expected (Grayson et al., 2007). Objects used in this chapter were checked for bias before use as to prevent any influence on the experiment (Grayson, 2012a). Significance was observed in the retention phase of the experiment in the vehicle treated animals ($P<0.01$) as vehicle explored the novel object more than the familiar. This significance was not observed in the sub-chronic PCP rats as they showed no preference for the novel object. This showed the PCP group failed to discriminate the familiar object from the novel and so both objects has an element of novelty for them. It has been shown that the animals will remain alert and want to explore any new object with only familiar needing re-examination. Any novel object when presented will be explored by "normal" animals until any novelty has been lost (Antunes and Biala, 2012) as is reflected in the significant ($P<0.05$) reduction in the DI. This kind of reduction on novel exploration has been previously seen in cognitively impaired animals by (Grayson et al., 2007). Line crossing data also showed no significant difference between the vehicle and PCP animals, suggesting that there was no effect on the animal's locomotor activity.

Behavioural work carried out in this chapter is supported by previous work in the field by Grayson (Grayson et al., 2007). Hooded Lister rats either dosed with vehicle (0.9% saline) or PCP (2.0 mg/kg bi-daily for 7 days) with 7 day washout

period. In all three experiments a PCP deficit is shown in NOR experimentation when compared to vehicle animals.

In support of the MRS data presented in this chapter we also found similar data post-mortem in the HPLC analysis of NAA in the same cohort of rats. A significant ($P<0.001$) reduction was seen in the sub-chronic PCP rats when compared to the vehicle. This reduction was of a similar size to that observed in the MRS data and so supporting the findings shown in the MRS section of this chapter. The association between the MRS and HPLC analyses, *in vivo* and *ex vivo*, is the first time this type of study has been completed when looking at the PCP model. At this current time there are no similar findings in the literature. The associations shown, being of a comparative change in NAA levels in both of the methods used and as such support the use of both methods for measuring NAA and its use as a biomarker for neuronal dysfunction in neurological diseases.

Since NAA being the largest signal marker in the healthy brain (Breiter SN, 1992) believed to be confined to neurons, a reduced level of NAA indicates a form of neuronal dysfunction or loss of neuronal structures (Bertolino et al., 2001) in patients with schizophrenia. This also supports the hypofrontality theory of schizophrenia. The combination of both the neurochemical NAA deficit and the behavioural deficits shown in this chapter, together with the supporting work in the field presents strong foundation for the use of NAA as a biomarker. Preclinical work could utilise these findings to investigate the effect of novel compounds on both the behavioural (e.g. NOR deficits) and pathological (i.e NAA) deficits with the aim of finding

compounds that restore both aspects. Because of the translational aspects of both these measures, a similar approach could also be utilised in clinical trials.

The main limitation in this experimental design was the low n numbers that we were able to scan with the MRS. Due to the high demand of the MRS scanning machine, cost of renting out the scanner and the length of time for setup and scanning of each rat, it reduced the time that we were able to scan. An increase in the number of rats scanned could lead to a tightening of the error bars and increase the significance of the decrease in NAA. The strength of the magnet can also contribute to the limitations of this experiment. As magnet power has increased so has the available resolution of the scanning techniques. Higher strength MRS scanners will allow smaller voxel sizes and enable us to look at NAA in more discrete regions in the rat brain

The findings in this chapter suggest that sub-chronic treatment with PCP shows a robust cognitive deficit at day 63 which is supported by the reduction of NAA in the MRS scanning *in vivo* and post mortem *ex vivo* HPLC analysis. With NAA already used widely in MRS scanning as a biomarker for neuronal integrity these findings show promising support for NAA being a viable biomarker for pre-clinical work into schizophrenia. Further work would increase the support for the findings with a larger n number as mentioned before as well as a greater time scale. Work into the decrease of NAA during PCP dosing would also show the steady decline of NAA and provide a better understanding of the relationship between chronic PCP dosing and neuronal dysfunction.

Chapter 4

Investigating levels of NAA in post-mortem tissue from controls and patients with Schizophrenia in both white and grey matter.

4.1 Introduction

Measurements of NAA using MRS have contributed important clues as to the pathophysiology of schizophrenia due to its association to brain function. Previous work done by (Marenco et al., 2006) has shown reductions in NAA in the medial temporal lobe and DLPFC (Dorsal Lateral Pre-Frontal Cortex). NAA levels being a seemingly heritable trait and covary with variations of the BDNF (Brain derived neurotrophic factor) and GRM3 (Metabotrophic glutamate receptor 3 gene) genes that may contribute to the cognitive dysfunctions that constitutes one of the cardinal features of schizophrenia. Most MRS papers that use NAA as a marker show small regional reductions in NAA or NAA / Cr (Bertolino et al., 1996; Callicott et al., 2000a; Deicken et al., 2001). One study to corroborate this evidence used MRS to compare NAA concentrations in 14 schizophrenics to 13 control subjects. NAA had been found to be decreased in the frontal lobe of affected patients when compared with healthy patient group (Tanaka et al., 2006). NAA reductions have been seen, using MRS, in the thalamus (Deicken et al., 2000; Ende et al., 2003; Jakary et al., 2005), anterior cingulate cortex (Deicken et al., 1997a) and cerebellum (Jakary et al., 2005). It has also been noted that an early sign of schizophrenia has been associated with a reduction of NAA or NAA/Cr. Examples of this can be seen in (Jessen et al., 2006), where a reduction in the NAA/CR was seen in the left frontal lobe of patients who were considered at risk for schizophrenia as well as in schizophrenics. Furthermore, a reduction in NAA levels in the dorsolateral prefrontal cortex of schizophrenic patients has been linked to a reduction in the performance in the Auditory Verbal Learning Task indication a connection with cognitive function and NAA levels (Ohrmann et al., 2007).

NAA and cognitive function – It has been reported from several different laboratories that greater NAA levels associated with better performance on certain types of cognitive testing, especially those that are broad measures of cognitive functioning (Ross and Sachdev, 2004). *In Vivo* measures of NAA always seem to be positively associated with increased cognitive function in normal individuals. It was reported that NAA concentrations form a left parieto-occipital white matter voxel predicted overall intellectual functioning in a young cohort of college aged subjects (Jung et al., 1999a). In the study above, NAA accounted for a 27%, variance in the IQ scores. It seems when compared to other biological markers of IQ, NAA seems to be one of the strongest indicators of intellectual functioning (Gray and Thompson, 2004). NAA levels have been directly linked to cognition in both the neurological diseases: Alzheimer's disease (AD) and schizophrenia. It has been seen that the ratio of NAA/Cr (Creatine) reduction is associated with the cognitive deterioration in studies in AD and has been used as a marker of progressing neurodegeneration (Ackl et al., 2005; Jessen et al., 2001; Jessen et al., 2000) in the hippocampus.

In neuroanatomical studies with schizophrenic patients a reduction in NAA concentrations in the frontal lobe was negatively correlated with the severity of negative symptoms and poor performance on the Wisconsin Card Sorting Test (WCST) in patients with chronic symptoms (Tanaka et al., 2006). It was shown in one study that a reduction in levels in the dorsolateral prefrontal cortex is negatively associated with disease duration (Molina et al., 2005), therefore suggesting that prefrontal NAA levels decrease progressively in schizophrenia. Reductions in the NAA/Cr ratios in the frontal and temporal lobes (Cecil et al., 1999) and hippocampus (Fannon et al., 2003) have also been observed. Therefore supporting

the widespread effect a neurological disease can have on the brain. Work done by (Hasan et al., 2011) points to a relationship between verbal memory and hippocampal integrity in schizophrenia patients which might be independent from deficits in other memory domains and that disturbed verbal memory functions in first episode schizophrenics might be linked specifically to hippocampal function. In isolation rearing models of schizophrenia (Harte et al., 2004) showed that NAA levels in the temporal cortex was reduced, also being supported by a pre-pulse inhibition test showing deficits in the isolate rats when compared to control.

The uses of antipsychotics have also been shown to have an effect on the levels of NAA in the dorsolateral cortex (Bertolino et al., 2001) and in the thalamus (Szulc et al., 2005). The paper by (Harte et al., 2005) can also show the effect of long term administration of the antipsychotic haloperidol caused increased (+23%) NAA levels in the striatum in the rat.

MRS is an important tool in the analysis of NAA in patient populations as it allows the levels of NAA to be measured in the same patients at a number of time points, with or without antipsychotics being present. Both afore mentioned studies (Bertolino et al., 2001; Szulc et al., 2005) used batches of patients in the absence of antipsychotics and in the presence of antipsychotics. Therefore providing a reliable result as the patients provide their own controls. Using these methodologies it is possible to measure levels of NAA over the course of an antipsychotic treatment regimen to see the neurochemical and pathological effect of antipsychotics.

Previous studies carried out by (Reynolds and Reynolds, 2011) has shown significant decreases in NAA in the hippocampus and superior temporal cortex (Nudmamud et al., 2003b) compared with control patients. Post mortem experimental reductions were shown in NAA that are comparative of schizophrenic patients, however the reductions seen were not as severe.

Taken together the studies above highlight some of the issues in MRS research whereby a number of conflicting results are reported, in terms of regions affected in schizophrenia studies. Alongside the difference in patient populations studied (differences include age, symptoms severity, medication status, disease duration etc) there are a number of methodological differences that must be recognised. These include differences in magnet strength, region of interest, voxel size, single-voxel vs. chemical shift imaging signal acquisition, post-processing methods approach to partial volume segmentation and how NAA is measured and reported.

4.1.1 Aim

- Investigate levels of NAA in the frontal cortex in post-mortem tissue from controls and patients with Schizophrenia in both white and grey matter,

4.2 Human Materials and Methods

4.2.1 Subjects

Brain tissues (frozen sections on slides stored at -80^{o}C) were provided by the Stanley Foundation Neuropathology Consortium. Samples were taken from the frontal cortex from patients with Schizophrenia and matched controls. Patient demographics are shown in table 10 (Full patient demographics are shown in the Appendix B chapter). Samples were from 32 matched subjects. There was no significant difference between the two groups in the study with regards to age, sex, post-mortem delay, brain PH, brain weight or storage time.

	Control	Schizophrenia
Demographic	n = 15	n = 17
Age (Years)	46.1 ± 8.6	40.1 ± 9.5
Gender	12 M, 3 F	14 M, 3 F
Post-mortem interval (Hours)	27.2 ± 14.0	27.5 ± 17.2
Brain hemisphere used (Right : Left)	10 : 5	9 : 8
Age of onset (Years)	N/A	21.8 ± 4.1
Duration of illness (Years)	N/A	18.4 ± 9.0
Race	15 White	17 White

Table 10: Demographic and clinical information for control and schizophrenic groups.

4.2.2 Sample Preparation

Frozen cortex brain sections, two per patient, were removed from -80^{o}C storage and placed onto dry ice. Each section in turn was taken and rapidly thawed and the white and grey matter was marked on the underside of the slide. Sections of white and grey matter were scraped using a razor blade into separate Eppendorfs. 60 µl of 0.1

M perchloric acid was added to the Eppendorfs. Samples were vortexed, placed on ice for 5 minutes and vortexed again. Samples were then placed into a centrifuge and spun at 12,000 rpm for 5 minutes. After centrifugation 50 µl of the supernatant was taken from the samples and placed into a second Eppendorf with 30 µl of 0.2 M sodium carbonate and 920 µl of 50 mM phosphate buffer. This sample was then placed into the SAX extraction columns and NAA was extracted following the same procedure as in chapter 2 of this thesis. The protein pellet was kept and used for the protein analysis.

4.2.3 Human Sample HPLC Experimental Overview

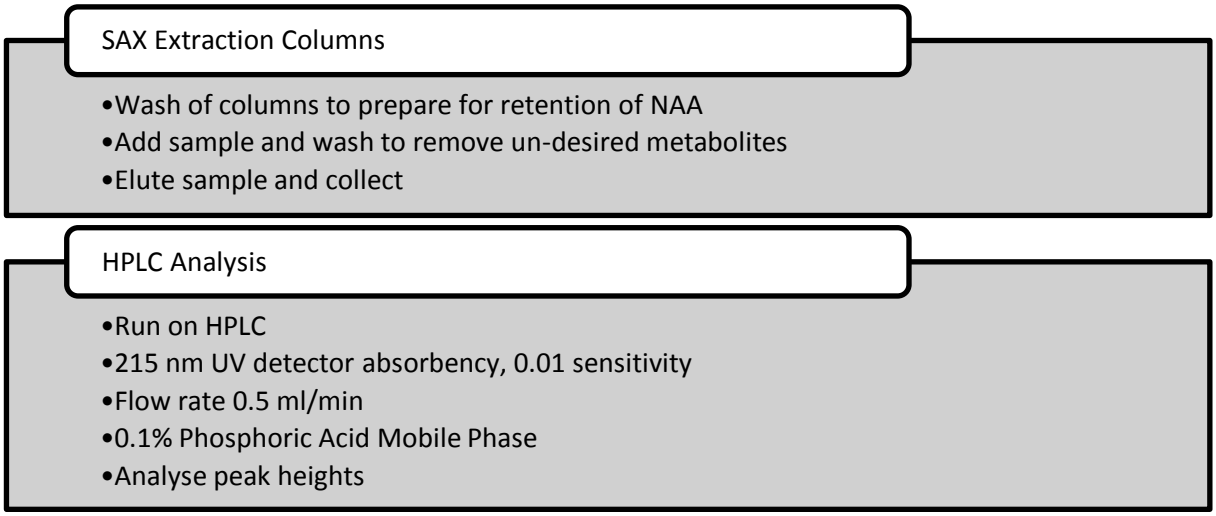

Table 11: Human Sample Experimental overview

4.2.4.1 HPLC Analysis of NAA

Please refer to chapter 2 (2.2.5 - 2.2.8) for the full HPLC methodology.

4.2.4.2 Protein Analysis

Unlike previous methods for HPLC analysis brain tissue weight could not be used as the samples from the slides were too small to weigh. Therefore, protein content from the sample was assessed by using the pellet collected during sample preparation. This was carried out by dissolving the pellet in 500 µl of 1 M sodium hydroxide and then processed using the coomassie blue method. All samples were prepared in a 1:5, 1:10 and 1:20 dilution in triplicate as well as one pure sample of the dilution into a 96 well plate (20 µl). A bovine serum albumin (BSA) solution (2 mg / ml in 1 M sodium hydroxide) was also prepared and then a serial dilution prepared in 1, 0.5, 0.25, 0.0625 and 0 mg / ml and again placed in triplicate into the 96 well plates (40 µl). To each of the wells 200 µl of the Bradford reagent was added and the plates were read at 595 nm. Protein values for all samples were determined in relation to the standard curves.

4.2.5 Quantification of NAA

Levels of NAA were calculated using the following formula:

NAA (nmol/mg protein) = ((0.03/standard peak height) x sample peak height x (6/5) x 1000) / (Protein value / 2)

4.2.6 Statistical Analysis

An ANOVA was used to analyse the data between controls and schizophrenics in the white and grey matter regions. A stepwise regression analysis was used to determine

any relationships between white and grey matter with control and schizophrenics grouped together. Following analysis no significant correlation was found between the white and grey brain matter in relation to; pH, brain weight, age, storage time, post-mortem interval, refrigeration interval and NAA levels.

The statistical analysis was completed on SPSS/PASW version 20. A p value < 0.05 was considered to be significant.

4.3 Results

Tables 16 and 17 show the patient raw data for both white and grey matter for both control and schizophrenic groups after calculation from the peak heights.

	NAA nmol / mg protein	
Patient TAC Codes	White Matter	Grey Matter
Tac 12	15.89	26.89
Tac 17	37.92	65.96
Tac 28	37.98	46.96
Tac 29	33.06	39.92
Tac 48	22.18	48.69
Tac 49	24.15	30.58
Tac 52	31.74	63.00
Tac 65	35.60	25.79
Tac 70	18.81	21.53
Tac 73	47.37	60.77
Tac 75	32.20	35.11
Tac 77	(Error with protein)	52.08
Tac 78	33.95	89.63
Tac 79	35.91	48.70
Tac 82	6.54	49.89
Average	**29.52**	**47.04**
SEM	**2.87**	**4.69**

Table 12: Patient data from Control groups. TAC 77 patient's protein analysis failed so NAA levels were unable to be calculated.

	NAA nmol / mg protein	
Patient TAC Codes	White Matter	Grey Matter
Tac 13	19.03	20.36
Tac 14	22.32	26.80
Tac 16	39.51	17.50
Tac 27	9.19	14.08
Tac 53	55.75	61.76
Tac 64	30.55	38.14
Tac 67	14.71	16.13
Tac 68	37.62	27.54
Tac 69	34.17	39.13
Tac 71	12.68	29.53
Tac 76	38.10	34.43
Tac 80	31.98	43.21
Tac 81	47.13	66.76
Tac 94	28.45	41.86
Tac 95	17.68	20.14
Tac 96	24.61	37.33
Tac 99	28.84	35.37
Average	**28.96**	**33.53***
SEM	**3.03**	**3.60**

Table 13: Patient data from schizophrenic groups. *P<0.05; Significant reduction in the levels of NAA (nmol/mg protein) in the grey matter of schizophrenic patients when compared to controls, one way ANOVA.

Analysis of the data using one way ANOVA presented significantly ($F_{1,30}$=5.346, P<0.05) lower NAA levels in the grey matter of schizophrenic patients when compared to control patients as shown in figure 28.

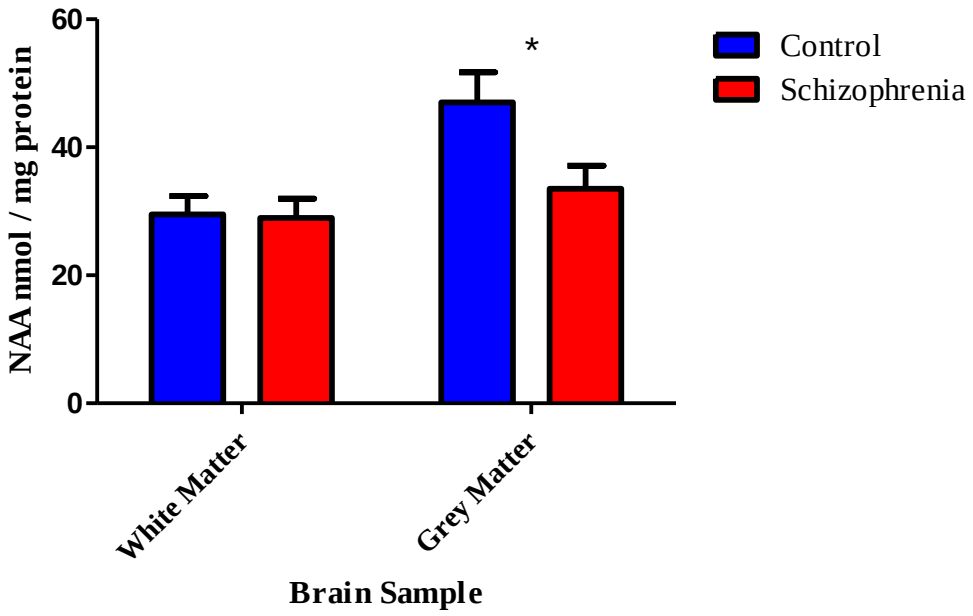

Figure 28: NAA levels in grey and white brain matter in control and schizophrenia patients. Data are shown as the mean ± SEM. (n= 15-17 per group). *P<0.05; significant reduction in the levels of NAA (nmol/mg protein) in the grey matter of schizophrenic patients when compared to controls, one way ANOVA.

Scatter plot of data showing range of values in both groups and the mean point of all the data points shown in figure 29.

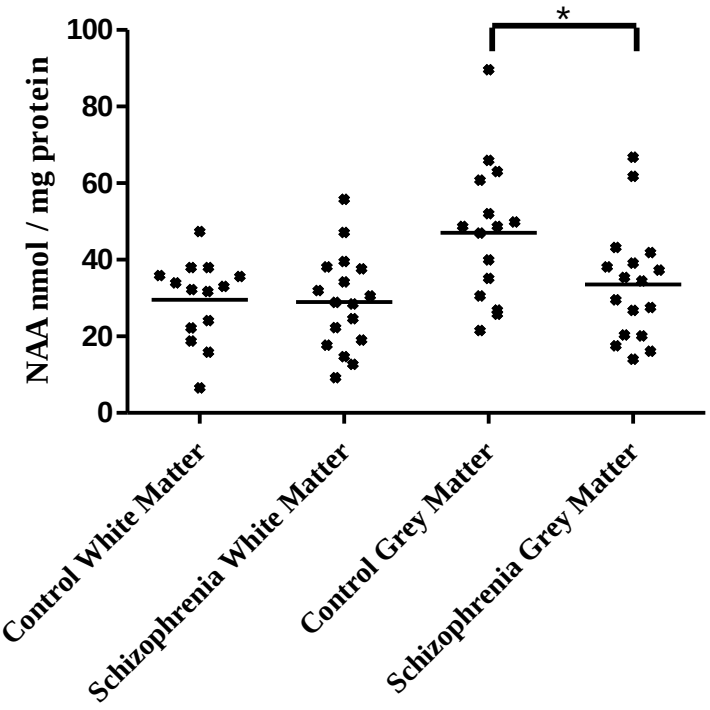

Figure 29: NAA levels in grey and white brain matter in controls and schizophrenia patients.. Data are shown as the mean with individual data points. (n= 15-17 per group). *P<0.05; significant reduction in the levels of NAA (nmol/mg protein) in the grey matter of schizophrenic patients when compared to controls, one way ANOVA.

4.3.1 Statistical Correlations

Comparison of overall NAA levels between the white and grey matter, combining both the control and schizophrenic groups, showed significant (P<0.01) difference as shown in table 14.

White Matter vs Grey Matter (Control + Schizophrenic groups)	0.007**

Table 14: Comparison data from white matter vs grey matter in both the control and schizophrenic groups combined. **P<0.01; significant difference between white and grey matter representing higher NAA levels in the Grey Matter, Student's t-test.

Comparison of white matter vs grey matter in the two separate groups showed significant (P<0.01) difference in the controls shown in table 15. There was no significance observed in the schizophrenic group using the same comparison.

Control White Matter vs. Grey Matter	Schizophrenia White Matter vs. Grey Matter
0.003**	0.086

Table 15: Comparison data from white matter vs grey matter in the control and schizophrenic groups. **P<0.01; significant difference between white and grey matter in the control group representing higher overall NAA levels in the control group, Student's t-test.

Within the groups (control and schizophrenic) and white and grey matter, a Pearson's correlation coefficient was used to determine any relationship between, pH, brain weight, age, storage time, post-mortem interval, refrigeration interval and NAA levels. In the schizophrenic group a correlation was also plotted against age of onset, duration of illness, lifetime antipsychotics and NAA levels. There was a significant (P<0.05) correlation between white matter NAA and post mortem interval in the schizophrenic group (table 16 and 17, figure 30).

	Control WM	Control GM	Schiz WM	Schiz GM
Brain pH	.289, P=.317	.113, P=.688	-.287, P=.264	-.048, P=.885
Brain Weight	.162, P=.581	.045, P=.874	-.196, P=.450	-.071, P=.787
Age	-.284, P=.581	.129, P=.647	.063, P=.812	-.142, P=.587
Refrigeration Int.	.349, P=.242	.109, P=.710	-.336, P=.118	-.019, P=.941
PMI	.357, P=.210	-.300, P=.277	-.557, P=.015*	-.281, P=.275
Age of onset			.349, P=.170	.138, P=.597
Duration			-.095, P=.716	-.214, P=.410
Lifetime Antipsychotics			.372, P=.141	.312, P=.222

Table 16: Pearson's correlation data from SPSS output. Data are shown as Pearson's correlation, significance. (n = 15 – 17 per group). *P<0.05; significant correlation between post mortem index and levels of NAA in white matter samples in schizophrenic patients, Pearson's correlation coefficient.

Patient TAC Codes	Post Mortem Interval (Hours)	White Matter NAA (NAA nmol / mg protein)
Tac 68	9	37.62
Tac 76	9	38.10
Tac 81	10	47.13
Tac 53	13	55.75
Tac 80	15	31.98
Tac 67	18	14.71
Tac 13	19	19.03
Tac 16	26	39.51
Tac 94	28	28.45
Tac 69	29	34.17
Tac 27	30	9.19
Tac 99	33	28.84
Tac 96	34	24.61
Tac 14	35	22.32
Tac 64	36	30.55
Tac 95	43	17.68
Tac 71	80	12.68
Average	27.2	28.96
SEM	14.02	3.03

Table 17: Raw data from Pearson's correlation from SPSS analysis.

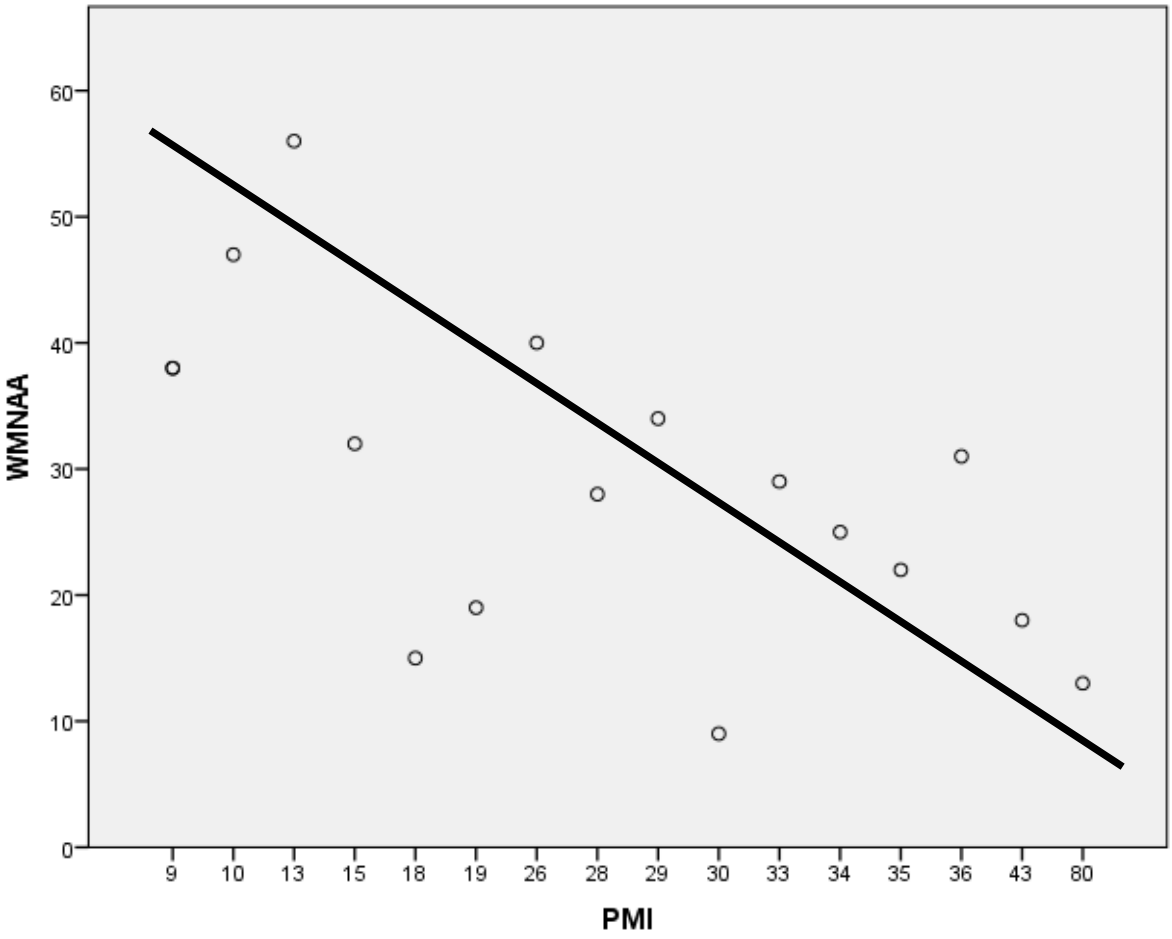

Figure 30: Showing the trend correlation between white matter NAA levels and the post mortem index in the schizophrenic group as shown from SPSS. Graph shows significant ($P<0.05$) negative correlation between NAA levels and the length of the PMI. Trend line shows linear correlation.

4.4 Discussion

As mentioned before in this thesis, NAA is found in neurons and axonal tissues however, not in mature glial cells therefore suggesting that a reduction in NAA is an indication of neuronal loss/dysfunction. This could be a factor of interneuronal neurophil, neuronal or axonal metabolic dysfunction or some combination of the aforementioned processes.

In this chapter we have shown that NAA deficits in the frontal cortex can be seen in post mortem grey matter tissue in schizophrenic patients when compared to controls. Significant ($P<0.05$) decrease in the levels of NAA in the frontal cortex grey matter samples, controls compared to schizophrenics, were observed (controls; 47.03 ± 4.69, schizophrenics; 33.53 ± 3.60). There was no observable reduction in the NAA in the white matter of the same samples (controls; 29.57 ± 2.84, schizophrenics; 29.05 ± 3.04). This leads us to believe that detectable neuronal dysfunction occurs in schizophrenic patients. These findings are consistent with the current literature in the wider scientific community. Reductions found in the frontal cortex are suggestive of disease neuronal pathology in this region which in turn may be related to the cognitive deficits seen in schizophrenic patients. Unfortunately there is no cognitive data for the individuals in the current study. Similar reductions have been shown previously in the work by (Molina et al., 2007). Molina and colleagues found a decrease in the levels of NAA in the DLPFC, using MRS scanning, in both the left and right hemisphere of schizophrenic patients when compared to controls. We also see a significant ($P<0.01$) difference in the level of NAA in the grey matter, with a combination of control and schizophrenic samples, when compared to the white

matter (Grey Matter; 39.86 ± 3.16, White Matter; 29.29 ± 2.04) showing a higher level of NAA overall in the neuronal cell bodies. Comparison showed that NAA levels in grey vs white matter in controls showed significant ($P<0.01$) difference when compared to each other (white matter; 29.57 ± 2.84, grey matter; 47.03 ± 4.69) showing that in normal brains higher levels of NAA are located in the grey matter. Subsequently, in the schizophrenic samples the same comparison showed no significant difference (white matter; 29.05 ± 3.04, grey matter; 33.53 ± 3.60), possibly reflective of deficits in grey matter in schizophrenia.

NAA levels have been shown to increase in schizophrenic patient brains following antipsychotic treatment (Bertolino et al., 2001) suggesting that NAA is a reflection of the dysfunction of neurons and not just an indicator of neuronal loss. The increase shown was seen with both typical and atypical antipsychotic medication when looking at the DLPFC. In chronic patients (illness duration 10.7 ± 5.9 years), increases in NAA levels following antipsychotic medication have also been seen in the work by (Fannon et al., 2003) again in patient brains at the time of medication. This increase in NAA levels was observed in both typical and atypical antipsychotic medication, although the effect was emphasised in the atypical groups. This increase due to medication was not observed in other regions such as the hippocampal area. The increase seen is theorised to be linked to the glutamatergic neurons. Therefore, a change in glutamate levels will affect NAA synthesis and concentrations (Cendes et al., 1997; Hugg et al., 1996; Tedeschi et al., 1996). An increase in glutamatergic metabolism following typical and atypical antipsychotics could convey an effect on NAA levels (Eastwood et al., 1996). However, other studies in the field have noted no effect on the levels of NAA following antipsychotics (Bustillo et al., 2008).

Work in animal models (Harte et al., 2005) showed that chronic treatment of haloperidol had no effect on NAA levels in frontal and temporal cortex, thalamus, hippocampus amygdala and the nucleus accumbens. There was an increase, however, in the striatal levels of NAA. Further work needs to be assessed using individual patient antipsychotic data to measure the specific long term effect of antipsychotics post mortem to determine the longevity of the reported NAA increases. With all of the previous research taken into account, it is possible to discount any relationship between antipsychotic treatment exposures. Therefore, the levels of NAA observed are not compromised by any drug action.

It was important to take into account all of the patient demographics in this chapter to make sure that effect on patient life was taken into account on the levels of NAA. We found only one correlation in this instance via Pearson's correlation on the levels of white matter NAA and PMI in the schizophrenia group. Brain degradation begins at patient death and so neurons will begin to degrade at this point and therefore the amount of NAA contained in these regions. It has been noted previously that NAA loss has not been seen for post mortem delays of up to 32 hours in the work by (Passani et al., 1997) in human brain tissue. However, the tissue that has been used in this thesis had a range of 9-80 hours in PMI. This finding was not observed in the control white matter or in the grey matter for each group. Furthermore, there was no significant differences between the groups in terms of PMI.

Recent work by Jessen (Jessen et al., 2013) also supports previous findings as his group shows a small reduction in NAA levels in the left frontal lobe of schizophrenic

patients. However, this small reduction did not show statistical significance. In a meta-analysis (Steen et al., 2005) there are reports of reduced NAA levels and grey matter in the frontal lobe in patients with schizophrenia. It is important to note that all work in the frontal cortex / lobe in the field is mostly done with MRS. Patient MRS can come across parameters that can call into question their results such as, voxel size and placement, field strength and patient characteristics.

Correlations between cognitive deficits and NAA in first episode schizophrenic patients have been observed (Galinska et al., 2007). There was a significant association between NAA reductions in the frontal cortex in comparison to the Wisconsin Card Sorting Test (WCST) showing that NAA can display a relation between cognitive functioning in first episode patients. Unfortunately, the same paper showed no association between the control NAA levels and the schizophrenics. This could be due to inconsistencies of NAA reductions in early stages of the illness and that NAA is negatively associated with the disease progression (Molina et al., 2005). This theory is also supported by (Tanaka et al., 2006) showing that NAA decreases in the left frontal lobe of schizophrenics with chronic status when compared with healthy controls. This finding was supported with positive and negative symptoms scale scores as well as WCST. This theory is tied together by the work by (Ohrmann et al., 2005) showing that NAA is reduced in chronic schizophrenic patients when compared to controls. However, no reduction in the acute schizophrenic patients again compared with controls. These findings further support the theory that chronic schizophrenia shows evidence of being a progressive brain disorder with neuronal function declining as the disease progresses from acute to chronic.

Previous work done in this thesis supports the findings in this chapter. Both in the MRS PCP animal model and the HPLC determination of NAA in a PCP rat show an association of reductions in the NAA levels in the frontal cortex. Not only does this evidence support the findings in this chapter but supports the PCP model as a representation of a model of aspects of schizophrenia. These chapters show significant support for NAA's use in schizophrenic models however more support is needed on another disease model to show NAA's use in the wider neurological diseases.

Post-mortem reductions in NAA have been seen before in the literature especially by the work done by (Reynolds and Reynolds, 2011). In relation to the work shown in this paper the following paper has shown similar work in this field and used a similar protocol (Reynolds et al., 2005). Reynolds showed that there was a significant difference in post-mortem tissues in the hippocampus, amygdala and caudate nucleus in schizophrenic patients when compared to control patients. However, this paper only used the grey matter for analysis, whereas we studied the white matter NAA concentrations alongside the grey matter.

White matter in this chapter was seen to have no significant difference between controls and schizophrenic patients. However, literature in this field using diffusion tensor imaging (DTI) (Du et al., 2013) showed that abnormal myelination and abnormal NAA diffusion was observed with an increase of NAA axonal diffusion

coefficient (ADC). An increase in NAA ADC has been linked with a lower education attainment as well that white matter abnormalities and axon geometry are related to the factors that relate to the functional outcomes of the disease. Similar findings were seen in the work by (He et al., 2012), again showing that NAA levels in the left frontal lobe white matter are decreased and this finding associates with the severity of negative symptoms, more severe positive symptoms and NAA/Cr ratios in the bilateral frontal lobes. Although the findings in the literature show compelling evidence that NAA is reduced in the white matter, a comprehensive meta-analysis of papers with 1209 controls and 1256 schizophrenic patients shows that although white matter NAA is shown to be reduced there was no overall evidence that white matter NAA is reduced in the frontal lobe in schizophrenic patients when compared to controls (Steen et al., 2005). This analysis reports that most studies are underpowered as the minimum sample of control and schizophrenic patients were not met to provide the required for acceptable statistical power. This provides support for the findings that we have shown in this chapter; that white matter is not reduced in the frontal cortex.

NAA has been shown in previous work to be a good indicator of neuronal loss. Work by (O'Neill et al., 2000) using MRSI and PET to show NAA losses have a direct correlation with neuron density in a study using 19 subjects (observed effect in 18 out of the total 19 patients). This study showed a linear correlation of increasing grey matter with grey matter NAA content and that the relationship of NAA to grey matter was found to vary with the cognitive status between subjects. These findings suggest that the metabolic activity increases with the quantity of the levels of NAA found in these neurons and that NAA can be a marker of a decrease in the metabolic

activity in disease states. Work by (Yamasue et al., 2002) has also been shown to support the aforementioned theories as well as the results shown in this thesis. Again, in this work there was an association between grey matter volume and NAA levels.

In conclusion, schizophrenia displays neuronal dysfunction in the frontal cortex that can be clearly measured and quantified using NAA as a biomarker. This in combination with previous works in this thesis supports the main hypothesis that NAA has strong ability to measure neuronal dysfunction in pre-clinical models and now in clinical subjects. The reductions in the pathology of NAA in the frontal cortex are characteristic of schizophrenia. The findings support, and are supported by, the earlier work detailed in previous chapters in this thesis. If this work was to be elaborated a larger cohort of patient tissue samples would be used as well as removing any PMI subjects over 32 hours to reduce any influence the PMI could have on the levels of NAA in the samples. Also further studies in other brain regions using the split of white and grey matter would be an interesting elaboration on the previous work by Reynolds (Reynolds and Reynolds, 2011) as well as looking into different neurological diseases. The recent study by Jarskog and colleagues highlight the potential use of NAA in preclinical and clinical settings for assessing novel treatment strategies for schizophrenia.

Chapter 5

Investigating levels of NAA in a preclinical model of relevance to Alzheimer's Disease.

5.1 Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that was initially reported by Alois Alzheimer in 1906 (Maurer et al., 1997). It is associated with severe memory loss, and cognitive and behavioural deficits (Youssef et al., 2008). Approximately 2-5% of cases arise from a familial form of Alzheimer's disease having an early onset before the age of 65 (Blennow et al., 2006). This is commonly seen in individuals with mutated forms of amyloid precursor protein (APP), presenilin 1 (PSEN1) or presenilin 2 (PSEN2) genes that have been passed down through their families (Raux et al., 2005). Most cases are termed sporadic with environmental (age) and genetic factors acting as risk factors. The largest genetic risk factor being a carrier of the APOEε4 allele (Strittmatter et al., 1993), of which between 40 – 80% of cases express one APOEε4 allele (Mahley et al., 2006). At present, AD affects approximately 800,000 people in the UK (Alzheimer's Society, n.d.) and in 2010, a study published in The Lancet (Murray et al., 2013) reported that AD was the tenth leading cause of death in the UK. It is a significant global challenge and the World Health Organisation (WHO) estimates that 35.6 million people worldwide are affected by AD (World Health Organisation, 2012).

5.1.2 Alzheimers Progression

The main features of AD are seen to be senile plaques (SP) and neurofibrillary tangles (NFT) (Nestler et al., 2009). SPs arise from the deposition of the extracellular 39-43 amino acid, **amyloid-β peptide** (Aβ) (Nestler et al., 2009) and NFTs arise

from the accumulation of the abnormally phosphorylated protein, tau (Nestler et al., 2009).

As AD advances, tissue shrinkage in the brain is prevalent and more severe in regions such as the hippocampus (de la Monte, 1989), which has a significant role in memory formation. The sulci or grooves in the brain surface are widened and the ventricles, which hold the cerebrospinal fluid (CSF) are enlarged (Figure 31). Morphological changes to the brains structure have been shown previously to be linked with decreases in NAA in regions that are predominantly affected by AD pathology (Schuff et al., 2002).

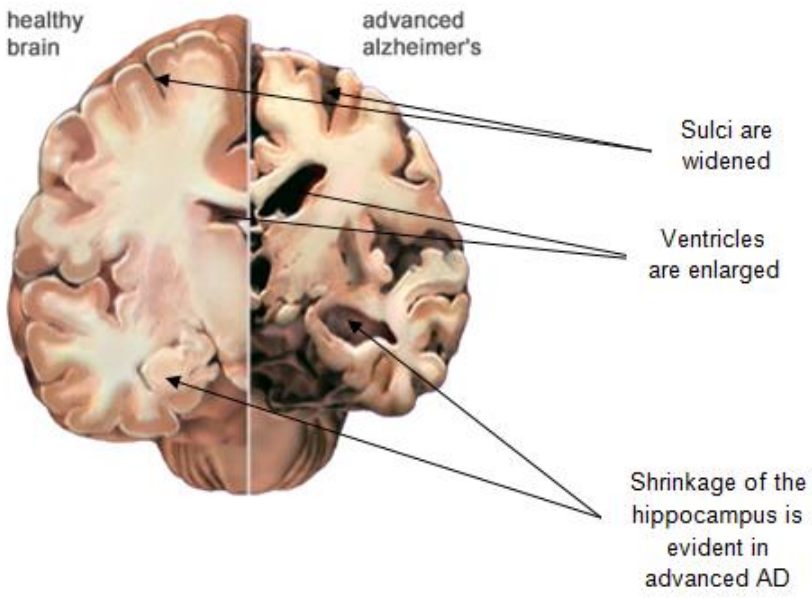

Figure 31: Comparison of brain changes in healthy and advanced AD patients. Reproduced and modified from the (Alzheimer's Association).

AD is characterised by three stages: preclinical (Morris, 2005; Sperling et al., 2011), mild cognitive impairment (MCI) (Petersen et al., 1999) and AD dementia (McKhann et al., 2011), which are each accompanied by their own set of symptoms,

as detailed in table 32. These changes become increasingly apparent as the disease progresses and individuals will experience a progressive decline in brain function over time.

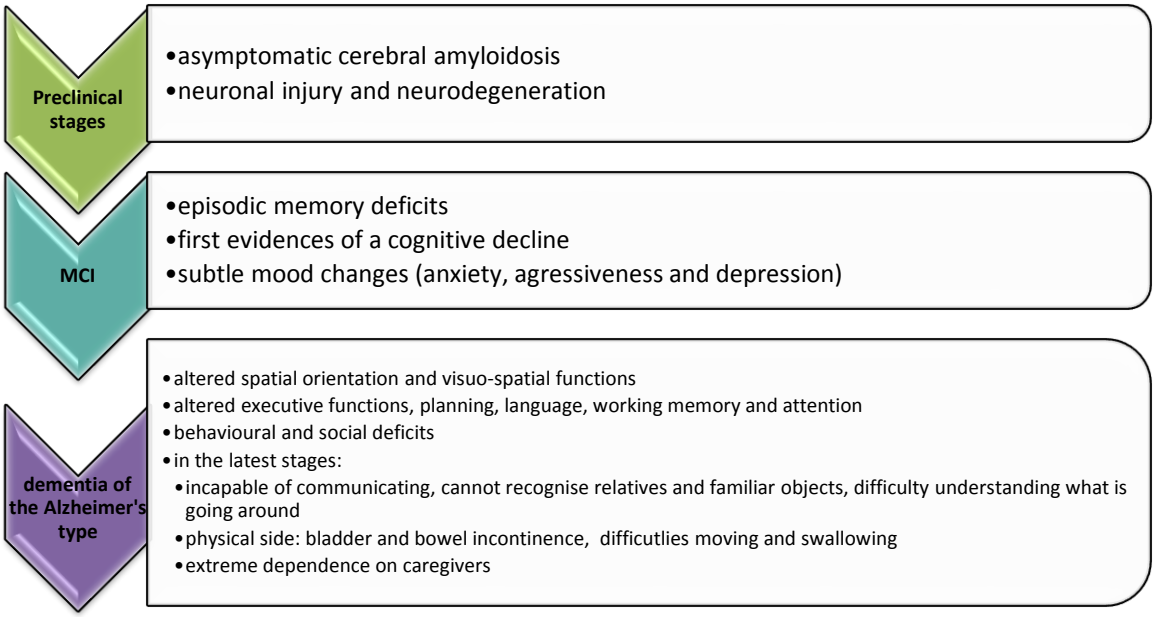

Figure 32: Stages of Alzheimer's disease progression

The preclinical stage will be the focus of this chapter as it is important to be able to identify the time point in the disease progression that has the highest chance to treat and prevent further disease progression. The model that we will use to look into AD progression is amyloid accumulation. We intend to use this model to investigate NAA's ability to be a marker to show at what point neuronal damage occurs.

5.1.3 Aβ peptide formation

Amyloid beta (Aβ), referred to as "the peptide from hell" (Zagorski et al., 1999), is composed of both hydrophobic and hydrophilic regions causing the peptide to naturally aggregate. Aβ peptide is formed when amyloid precursor protein (APP) is processed through the amyloidogenic pathway (Yamada and Nabeshima, 2000), and this forms the starting point for the amyloid cascade hypothesis (Hardy, 2006; Hardy and Higgins, 1992) (See Figure 33). APP is a transmembrane protein, which is widely expressed in human tissues (Nestler et al., 2009). The APP ectodomain is cleaved by β-secretase or BACE 1 (β-secretase APP cleaving enzyme 1) to form sAPPβ and the β C-terminal fragment (βCTF/C99)(Yamada and Nabeshima, 2000). This is followed by the multiprotein γ-secretase, which cleaves βCTF/C99 to form a 40-residue Aβ peptide ($A\beta_{1-40}$) or a 42-residue Aβ peptide ($A\beta_{1-42}$) and the APP intracellular domain (AICD)(Zhang, 2012). $A\beta_{1-42}$ has a greater hydrophobicity and therefore tends to form aggregates a lot earlier in neurons than $A\beta_{1-40}$ (Nestler et al., 2009). For this reason, $A\beta_{1-42}$ is more toxic and is mainly associated with AD pathology (Nestler et al., 2009). Excess $A\beta_{1-42}$ production results in aggregates of small soluble oligomers. Large insoluble fibrils can also be generated, which lead to Aβ plaques (Nestler et al., 2009). Aβ aggregates consequently result in plaque formation and neuronal loss, a key hallmark in AD pathology (Nestler et al., 2009).

APP can also be metabolised through the non-amyloidogenic pathway, resulting in the cleavage of APP by α-secretase (Zhang, 2012) to form sAPPα and the α C-

terminal fragment (αCTF/C83)(Yamada and Nabeshima, 2000). αCTF/C83 is then cleaved by γ-secretase, to form the p3 peptide(Zhang, 2012) and AICD (Yamada and Nabeshima, 2000).This pathway prevents the formation of toxic Aβ peptides.

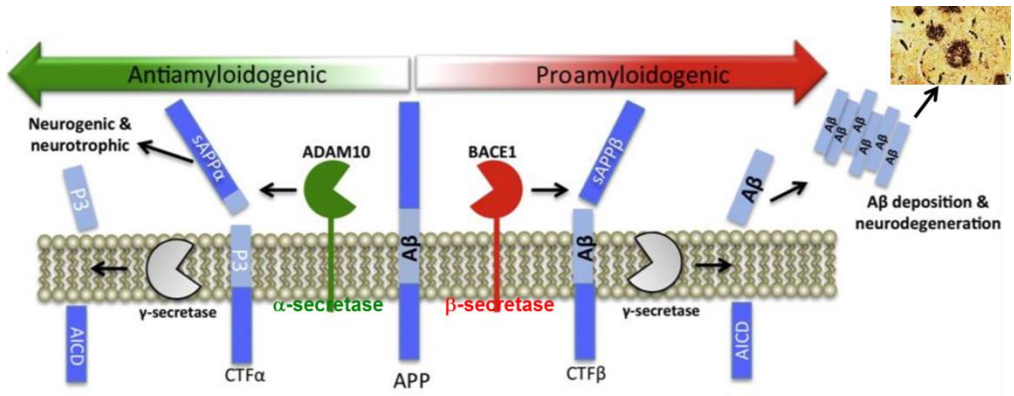

Figure 33: Intervening in the amyloid cascade.

The cascade is initiated when the large transmembrane protein APP is sequentially cleaved by the proteases β- and γ-secretase. The γ-secretase cleaves at several positions to generate Aβ peptides of different length. The 42 amino acid version, $A\beta_{42}$, is considered the most pathogenic species. Aβ is secreted into the extracellular space, where it aggregates, and Aβ aggregates may also form within cells. Cofactors like Zn and glycosaminoglycans (GAG) are proposed to enhance Aβ aggregation, and their interaction with Aβ is targeted by certain aggregation inhibitors. The aggregates ultimately form the signature plaques of Alzheimer's disease. The amyloid cascade hypothesis postulates that soluble aggregation intermediates, protofibrils or plaques trigger a cascade of events ultimately leading to neuronal loss and dementia. Several classes of amyloid therapeutics and the major concerns surrounding them are indicated.

5.1.4 Amyloid Cascade

The first amyloid cascade hypothesis was originally proposed by (Hardy and Higgins, 1992) and has been very significant to the field of research in AD. It postulates that the accumulation of Aβ peptide in plaques found in the brain initiate a series of detrimental cascading events (Pimplikar, 2009), which eventually will lead to AD dementia. Although supported by much evidence, this hypothesis has received scepticism from many scientists because it neglects to explain the poor relationship between the Aβ deposits in the brain and the cognitive deficits seen in individuals

(Youssef et al., 2008). Also, older individuals who remained healthy and cognitively intact throughout their lives were found to have many diffuse Aβ deposits during post-mortem examinations (Haass, 2010).

In light of this, (Haass, 2010) states that plaque burden is not associated with a decline in cognition (Haass, 2010). In a study conducted by Holmes et al. (2008) it was also found that plaque removal did not improve cognitive function (Holmes et al., 2008). These plaques are postulated to have a protective effect that slows neurodegeneration down, so removing them could be damaging for the individual (Lee et al., 2004). A modified version of the original hypothesis has since been developed and posits that the detrimental events that led to AD are due to Aβ and a number of other factors (Pimplikar, 2009). Therefore, solely preventing the effects of Aβ will not be effective in the majority of cases (Pimplikar, 2009). Theorised prevention methods focused on inhibition of the Aβ generation to prevent the full cascade effect as is shown in the figure 34. The other difficulty in such an approach is identifying patients in the early stages of the illness to test the appropriate treatments. In light of this we still need to further understand some of the early mechanisms underlying the initial events that precede plaque formation and neuronal loss.

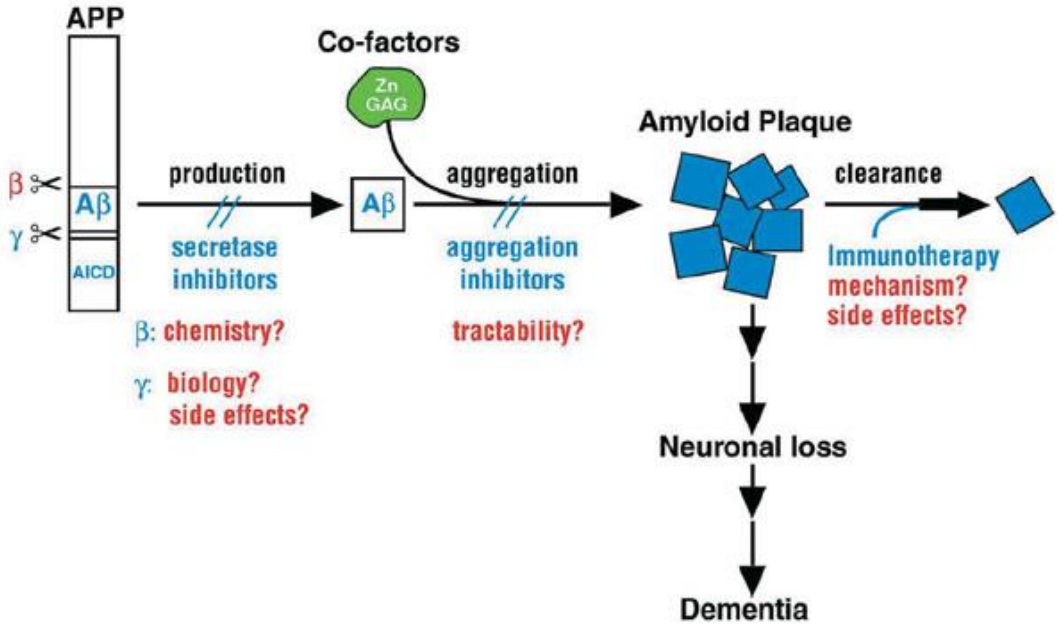

Figure 34: Shown is the hypothetical pathogenic cascade with potential therapeutic approaches. Immunotherapy aims to clear freshly secreted Aβ, Aβ aggregates and Aβ deposited in plaques. Image taken from (Citron, 2002).

New research efforts focus on oligomeric forms of Aβ or Aβ-derived diffusible ligands (ADDLs) (Ferreira et al., 2007), which are postulated to be the earliest, key toxic species in AD (Youssef et al., 2008). ADDLs are thought to result in synaptic dysfunction and learning and memory impairments, which are posited to occur before plaque formation. These oligomers are small enough to diffuse across the synaptic cleft and are able to disrupt cell membranes and lipid bi-layers (Braidy et al., 2012). They are thought to bind tightly at mature synapses (Hefti et al., 2013), where they will most probably bind to highly selective excitatory post-synaptic receptors (Ferreira et al., 2007). This binding action leads to synaptic dysfunction, which results in memory impairments due to Aβ induced deficits in long-term potentiation (LTP) and long-term depression (LTD) in the hippocampus (Cleary et al., 2005; Ferreira et al., 2007). In the present chapter we aim to use this model to effectively mimic the mechanism of action of Alzheimer's in rats in an early stage of

the disease development with post-treatment analysis with NAA. This methodology is outlined in the animal models section below as developed by previous research groups.

5.1.5 NAA and Alzheimers

A reduction in NAA concentration and NAA-creatine ratio has been seen to be a correlation in patients who test positive for clinical dementia. This is shown in Alzheimer's disease showing a 15 - 20% reduction in NAA - NAA/Cr ratio in the posterior cingulate gyrus grey matter (Kantarci et al., 2003; Moats et al., 1994; Shonk et al., 1995; Waldman et al., 2002). It is with the work by (Raichle et al., 2001) that the loss of NAA in the posterior cingulate gyrus in the early stages of Alzheimer disease supports the notion that this brain region plays an important role in attention and memory. Although the hippocampus is most implicated in memory and site of the earliest plaque deposition it is more technically difficult to define any significant loss of NAA using MRS due to technological limitations of *in vivo* MRS measurements. However use of a more robust MRS technique, long echo time chemical shift imaging, has shown there is a loss of NAA from the hippocampus in Alzheimer disease proportional to the volume loss of that structure (Schuff et al., 2002). In Schuff's paper his team were able to correctly determine the correct diagnosis in 80% of Alzheimer patients when compared to control when looking at NAA reductions. This value increased to 90% when the volume reductions were combined with the NAA data. This has also been seen when looking at the early stages of Alzheimer disease in the recently defined syndrome of mild cognitive

impairment. The progression of patients with MCI to AD is accompanied by a reduction in brain NAA/Cr levels (Kantarci and Jack, 2003; Kantarci et al., 2000; Kantarci et al., 2002). One study has reported that general NAA levels increased in Alzheimer disease brains (Harris et al., 2006) suggesting a compensatory up regulation of NAA synthesis in the remaining functional neurons. However, this up regulation of NAA would only be seen in brain areas where neuronal function was "normal".

5.1.6 Animal Models

Transgenic models are centred on the APP, PSEN1 and PSEN2 genes and are representative of familial AD. This, however, can be a limitation as only 2-5% of AD cases are in relation to familial AD (Lecanu and Papadopoulos, 2013). Therefore the larger majority of AD patients with the sporadic form of the disease are not covered by the transgenic models (Lecanu and Papadopoulos, 2013) but the models are still widely used in research to help us understand the progression of the disease and relate the underlying pathology to the behavioural deficits (Braidy et al., 2012). Due to the current treatments failing in the clinical setting it was recognised that alternative models had to be developed to better test novel compounds. The work done by (LaFerla and Green, 2012) shows that the transgenic models can show a decline in cognition and memory impairments prior to the formation of the Aβ plaques causing a focus on the Aβ oligomers. This was a significant turn in the development in the AD model as plaques occur before substantial cognitive deficits

are seen in humans as shown in the graphical representation of AD development as seen in figure 35.

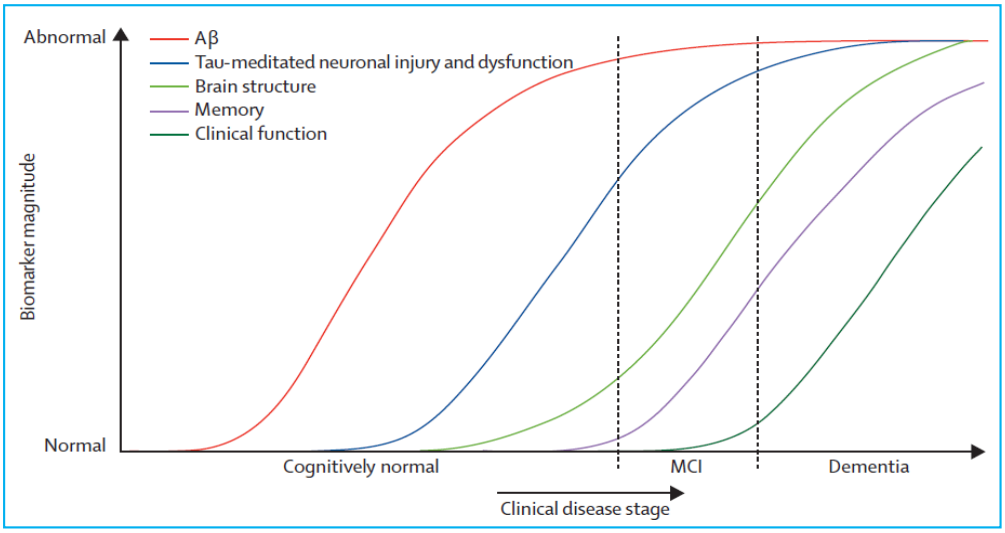

Figure 35: Alzheimer's disease progression in relation to biomarker levels (Jack et al., 2010)

Research in the field have shown increasing focus on the early stages of AD with the hope that effective interventions can be developed for better translational ability across to humans (Lecanu and Papadopoulos, 2013; Van Dam and De Deyn, 2011). Because of this, more work has been carried out on the amyliodogenic pathway with the use of small soluble Aβ oligomers in both acute and chronic administration models using an intracerebroventricular (ICV) injection of Aβ. In general the chronic models focus on slow administration through brain cannulation over a 4 week period (Lecanu and Papadopoulos, 2013; Van Dam and De Deyn, 2011). The acute model focuses on single ICV injection of AB oligomers to investigate the early underlying mechanisms that lead to synaptic and long term cognitive deficits in the animals.

In the current study we will be using an acute dose of small (monomers and tetramers), stable, soluble $A\beta_{1-42}$ oligomers to look into the early stages of the disease and changes in NAA levels and comparisons to behaviour. Studies with these oligomers have reported previous success at showing long lasting behavioural deficits in mice (Bouter et al., 2013) comparable to Alzheimer's memory dysfunction. Acute administration of small soluble Aβ oligomers has previously been shown to cause inhibition of long-term potentiation and enhancement of long-term depression from the early stages of AD (Lambert et al., 1998; Shankar et al., 2008; Wang et al., 2002). Alterations of metabolic pathways of AMPA receptor trafficking and NMDA receptor mediated axonal transport (Gu et al., 2009) leading to a reduction in synaptic plasticity and memory deficits (Decker et al., 2010) have also been reported. However the mechanisms that Aβ oligomers act upon, in early synaptic disruptions, are not fully understood and whether this involves any neuronal degeneration is unclear. We will utilise the neuronal marker NAA to investigate any changes in NAA in the brain following ICV administration of $A\beta_{1-42}$ oligomers

5.1.7 Aims

To determine if an acute dose of $A\beta_{1-42}$ oligomers have an effect on the behaviour of the animals by using the visual and learning memory test NOR. Follow onto post mortem analysis of the brains using HPLC to measure NAA levels in the brain to determine if NAA can be used to observe any neuronal loss/synaptic degeneration.

Combining these two experiments will provide better insight into the early stages of AD progression and the relation to NAA being used as a viable biomarker.

5.2 Methods

5.2.1 Animals

This study used 20 adult female (230 – 250 g) hooded-Lister rats (Charles River or Harlan, UK). Rats were housed (4-5 per cage) in a two tiered solid floored plastic cages by tecniplast (462 x 403 x 404 mm) containing sawdust, paper sizzle nest (Datesand, Ltd., Manchester, England) and fun tunnels (plastic environmental enrichment tubes., Datesand, Ltd., Manchester, England). Food (Special Diet Services Ltd., Essex, England) and drinking water was available *ad-libitum* in the home cage. The rats were disturbed only for cleaning which consisted of changing the cage once per week, weighing and experimental procedures listed below. Rats were housed in a single sex colony which was maintained under a constant temperature of approximately 21 ± 1 °C and humidity (40-50 %) under a 12h light:dark cycle (lights on at 0700 h). All experiments were conducted during the light phase and were carried out between 0900 h and 1630 h. All studies were compliant with the Animal Scientific Procedures act (1986) and approved by the University of Manchester Ethical Review Process.

5.2.2 Experimental procedure timeline

Shown in figure 36 is the general experimental procedure timeline outlining the timescale for the whole experimental procedure as detailed in this chapter.

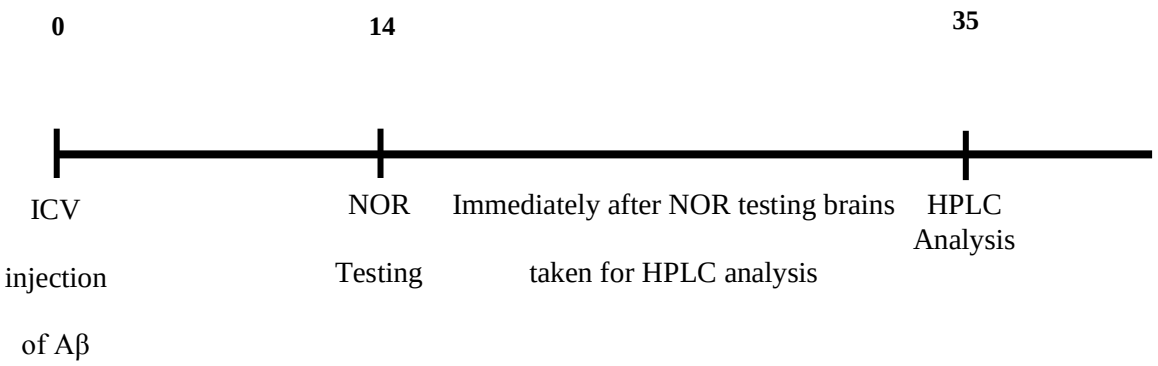

Figure 36: Alzheimers experimental procedure timeline

NAA measured was taken at day 14 and stored in the freezer until use on day 35 of the experiment.

5.2.3 Aβ Oligomers

Aβ $_{1\text{-}42}$ Oligomers were acquired from SynAging (www.synaging.com) and prepared as stock solutions of 0.5 mM in sterile 0.1 M phosphate buffered saline (pH 7.4) and stored in aliquots at -20^{o}C until needed. Previous analysis of the Aβ oligomers received has shown the oligomers to be essentially small trimers and tetramers (Bouter et al., 2013).

(http://www.synaging.com/en/Disease_Models/Alzheimers_Dementia/In_vivo_models.php) (Desbene et al., 2012).

5.2.4 Intracerebroventricular injection of Aβ oligomers

Rats were split into two groups randomly via cages and assigned to either receive intracerebroventricular (ICV) administration of Aβ oligomers (5 nmol in 10 µl/rat) or vehicle (10 µl saline/rat). Administration of the oligomers was achieved via surgical implantation. Briefly, animals were anaesthetised using a 4% isoflurane 0_2 mix in a gas chamber and then transferred to the surgical area. The rats head was shaved using an electrical clipper and then mounted to a stereotaxic frame where the level of isoflurane was decreased to 2%. An iodine alcohol solution (iodine 2.5% w/v and potassium iodide 2.5% w/v) was used to sterilise the prepared skin of the rat's head. A dose of analgesic was administered subcutaneously (Vetergesic® buprenorphine 0.3 mg/mL) at a dose of 0.1 mg/kg. A small amount of an ophthalmic ointment (Lacri-lube) was used on the rats eyes to prevent drying during the surgical procedure. Opening of the scalp was done with a midline sagittal incision and the skin held back using clamps.

Left ventricle positioning was found using the coordinates from bregma according to Paxinos and Watson (George Paxinos, 2007) (H_v = H - 0.8; Tr_v = Tr - 1.5). Piercing of the skull was achieved using a hand held drill. Steriotaxic administration of the oligomers or vehicle to the ventricle was done by lowering the needle to the coordinate (V_V = V - 4.5). Injection was done by a 10µl Hamilton syringe at a 5 µl / 2 min flow rate.

After injection of the oligomers the incision site in the scalp was closed and sutured. Rats were given an i.p. injection of saline to replace any lost fluids during surgery and to prevent dehydration during recovery. Rats were then placed into a surgical recovery incubator for 30 minutes whilst recovering from anaesthesia and then replaced to their home cages. Rats were observed for 4 hours post-surgery to make sure of a full recovery.

5.2.5 Novel Object Recognition Experimental Protocol Apparatus

Please refer to chapter 3 (3.2.6 - 3.2.8) for the full NOR methodology.

5.2.6 Brain Dissection

At the end of the experiments animals were sacrificed by CO_2 inhalation. Brains were quickly removed and the frontal cortex (anterior to bregma +3.20), striatum (bregma +1.20 to -0.20), hippocampus (dorsal: between bregma -2.30 to -3.80; ventral: bregma -4.8 to -6.04) and temporal cortex (bregma -2.30 to -3.80) were collected ((Paxinos and Watson, 1998) See figure 5). The analysis included bilateral hemispheric dissection. Dissected tissues were immediately frozen on dry ice and stored at -80 for further analysis.

Frozen sections were then weighed and 10 times their volume of 0.1 M perchloric acid was added. Tissues were homogenised to precipitate proteins and centrifuged for 5 min at 12,000 *x g* to form a pellet (Eppendorf Centrifuge 5415 C). The supernatant was removed and used to prepare the samples for analysis. Samples

consisted of 50 µL of supernatant added to 920 µL of 50 mM phosphate buffer and 30 µL sodium carbonate. Samples were extracted using anion exchange columns (Figure 5).

5.2.7 HPLC Analysis

Please refer to chapter 2 (2.2.5 - 2.2.8) for the full HPLC methodology.

5.2.8 General Aβ Oligomer Experimental Work Plan

Shown in figure 37 is the general work plan for the experimental procedures carried out in this chapter. Detailed instruction of the procedures can be found in previous chapters as listed above. The experiments in this chapter followed the order listed in the work plan.

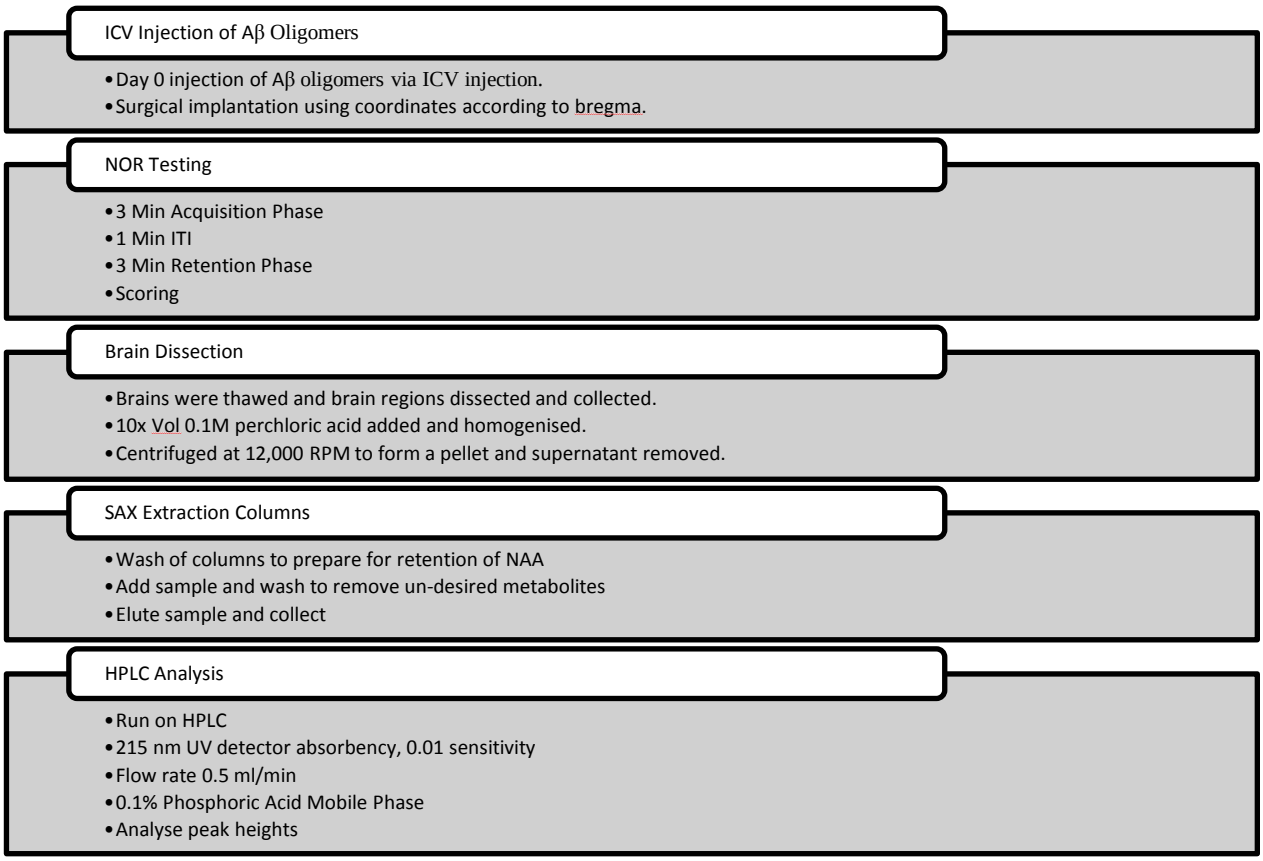

Figure 37: Experimental work plan

5.3 Results

5.3.1 NOR Results

Analysis of the data with a Student's t-test showed that treatment with Aβ oligomers did not produce any significant effect in the acquisition phase of the NOR experiment (Vehicle; t(9)=0.935, PCP; t(8)=0.1.705, NS; figure 38). In both treatment groups there was no significant difference in exploration time between the test animals, i.e. oligomer-treated animals, and control animals.

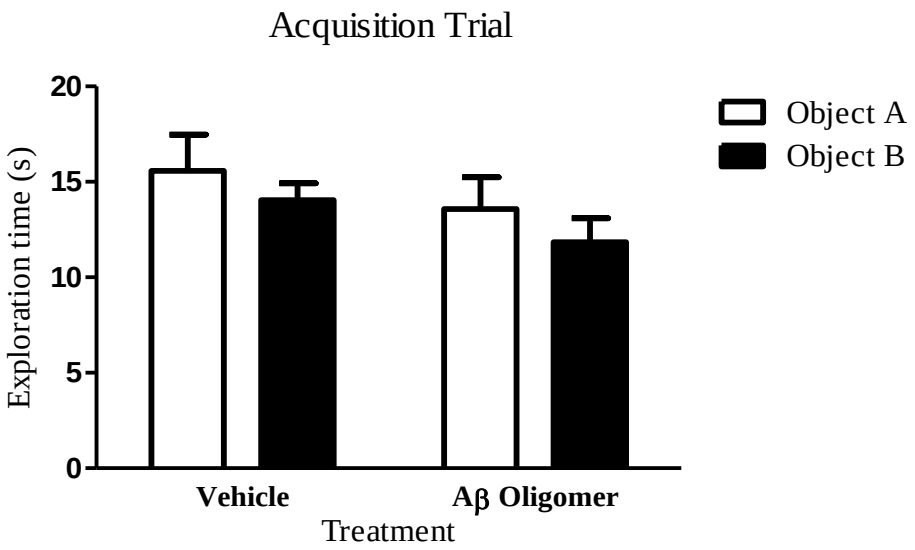

Figure 38: The effect of Aβ oligomer treatment (5nmol Aβ Oligomers in 10 μL, ICV) or vehicle (10 μL, ICV) on exploration times (s) of identical objects in the 3 min acquisition trial in the NOR test in female rats. Data are shown as the mean ± S.E.M. (n = 9 - 10 per group).

Analysis of the data with Student's t-test in the retention phase shows that treatment with Aβ oligomers produced no significant effect in the retention phase of the NOR experiment ($t(8)=-0.890$, NS; figure 39). Whereas the vehicle rats significantly ($t(9)= -4.923$, $P<0.001$) explored the novel object more than the familiar object following a 1 min ITI.

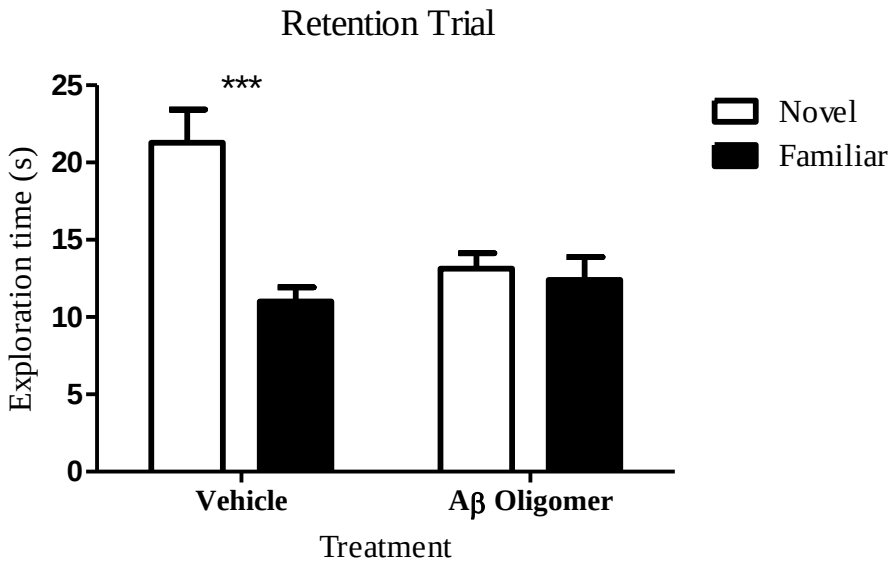

Figure 39: The effect of Aβ oligomer treatment (5nmol Aβ Oligomers in 10 µL, ICV) or vehicle (10 µL, ICV) on exploration times (s) of identical objects in the 3 min retention trial in the NOR test in female rats. Data are shown as the mean ± S.E.M. (n = 9 - 10 per group). ***P<0.001; significant difference between the time spent exploring the familiar object with the novel object, Student's t-test.

Discrimination index (DI) shows a direct ratio of exploration to remove total exploration time from the factor allowing direct comparison between the groups. Higher values representing good exploration of the novel over the familiar object. DI data showed a significant ($P<0.01$, figure 40) effect in Aβ oligomer-treated animals when compared with the vehicle group on the rats' ability to discriminate between novel and familiar objects in the retention phase following 1 min ITI.

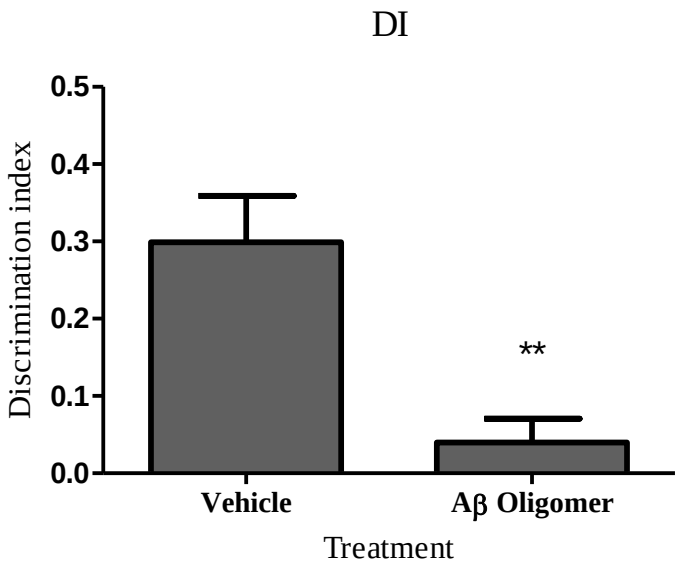

Figure 40: The effect of Aβ oligomer treatment (5nmol Aβ Oligomers in 10 μL, ICV) or vehicle (10 μL, ICV) on the discrimination index in the NOR test in female rats following 1 min ITI in the holding cage. Data are shown as the mean ± S.E.M. (n = 9 - 10 per group). *P<0.01; significant reduction in DI when compared to vehicle, Students t-test.

Line crossings (as a measure of locomotion in the test) showed no significant (Figure 41) effect of the Aβ oligomers when compared with vehicle following Students t-test in the acquisition and retention trial in the NOR test.

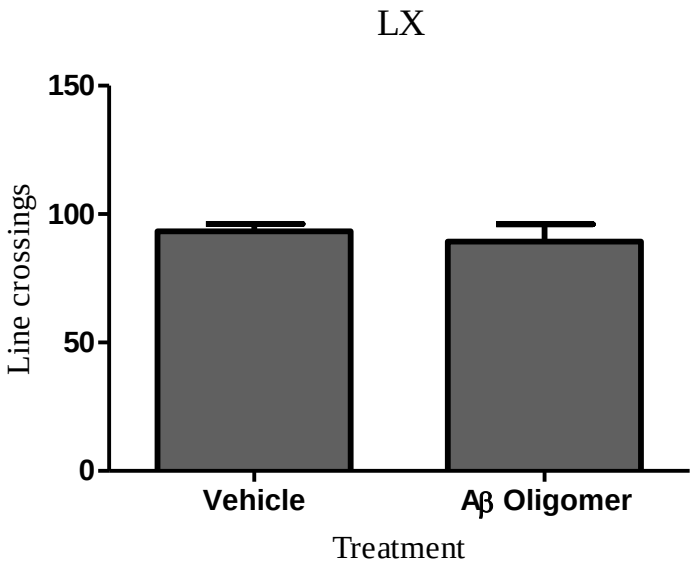

Figure 41: The effect of Aβ oligomer treatment (5nmol Aβ Oligomers in 10 µL, ICV) or vehicle (10 µL, ICV) on the total number of line crossings in the acquisition and retention trial in the NOR test in female rats following a 1 min ITI in the holding cage. Data are shown as the mean ± S.E.M. (n = 9 - 10 per group). No significant reductions following Students t-test.

5.3.2 NAA Results

Analysis of the data using one way ANOVA presented no significance in all brain regions, when comparing Vehicle with Aβ oligomer animals. ANOVA frontal cortex ($F_{1,9}$=0.024, NS; Figure 42), prefrontal cortex ($F_{1,8}$=1.305, NS; Figure 42), striatum ($F_{1,9}$=3.836, NS; Figure 42), temporal cortex ($F_{1,9}$=3.391, NS; Figure 42), dorsal hippocampus ($F_{1,9}$=0.120, NS; Figure 42) and ventral hippocampus ($F_{1,9}$=0.845, NS; Figure 42).

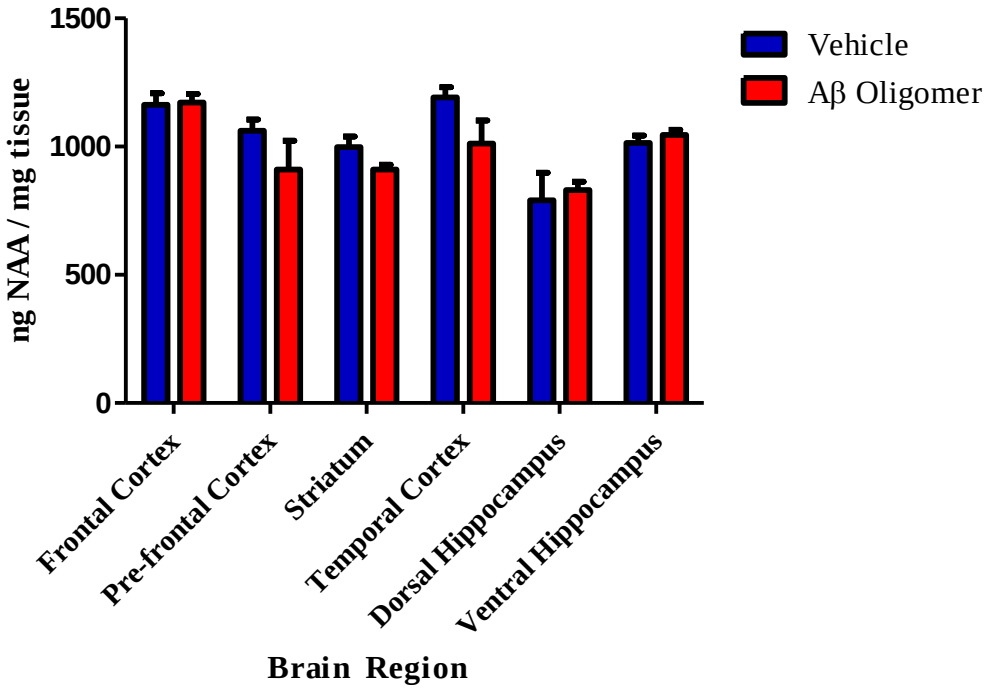

Figure 42: The effect of Aβ oligomer treatment (5nmol Aβ Oligomers in 10 µL, ICV) or vehicle (10 µL, ICV) on the levels of NAA in different brain regions using HPLC. Data are shown as the mean ± S.E.M. (n=4 – 5 per group). No significant reductions in NAA following one way ANOVA.

5.4 Discussion

Early cognitive decline in AD is thought to be a synaptic failure that occurs due in some way to soluble Aβ oligomers (Lacor et al., 2004). Over extended periods of time Aβ oligomers change to form a fibrillar form (Chromy et al., 2003) which, when bound to neuronal synapses, can inhibit LTP which is thought to be an important factor in learning and memory functioning. This process in turn can cause a decline in synaptic efficiency leading to the cognitive symptoms seen in AD. In this chapter we use small soluble $A\beta_{1-42}$ oligomers to mimic the effects of the early stages of Alzheimer's disease. Including, the decline in cognitive functioning observed in early stage Alzheimer's disease patients. We aimed to use this model to see if there was an observable behavioural deficit and if NAA could be used to show synaptic degeneration.

In the current studies our results showed that vehicle animals can correctly discriminate between novel and familiar objects following a 1 min ITI whereas following acute administration of our small soluble Aβ oligomers the rats demonstrated significant deficits in the NOR task. However, subsequent analysis of the brains demonstrated no changes in the levels of NAA in any of the brain regions showing that there is little or no neuronal loss at this early stage of the disease's pathology. The data showed that it is possible for the Aβ Oligomer treated rats to display a cognitive deficit at day 14 after administration (Cleary et al., 2005). However, there was no significant effect on the pathology of the brain using NAA as a biomarker. These cognitive deficits are thought to be caused by the Aβ oligomers causing impairment in cognitive functioning in the rats thought to be caused by the

neurodegenerative effect of the Aβ oligomers. The display of significant cognitive deficits, in transgenic mouse models, lends support that a patient with Alzheimer's disease express subtle cognitive changes in early stages of the disease's progression (Klein et al., 2001; Selkoe, 2002). Then, as the disease progresses, display the full symptomatology of Alzheimer's disease. This could be why pharmacological treatments for AD have higher efficacy in the early stages before the larger pathological damage has occurred.

In this chapter we found no significant difference in exploration times of the identical objects in the acquisition phase of the NOR task in both Vehicle and Aβ treated animals. Objects used in this chapter were checked for bias before use as to prevent any influence on the experiment (Grayson, 2012b). Taken together with the locomotor data it demonstrates that the animals treated with Aβ oligomers were capable of carrying out the task and had no overt behavioural disturbances. In addition both groups demonstrated no difference in weight gain following surgery (data not shown).

Significant differences between the groups were only observed in the retention phase of the experiment where vehicle animals explored the novel object more than the familiar one ($P<0.001$). This significance was not observed in the Aβ treated animals showing that these animals showed no preference for the novel object. This showed the Aβ oligomer animals failed to discriminate the familiar object from the novel and so both objects became novel for them. It has been shown that the animals

will remain alert and want to explore any new object with only familiar needing re-examination. Any novel object when presented will be explored by "normal" animals until any novelty has been lost (Antunes and Biala, 2012). This kind of reduction on novel exploration has been previously seen in cognitively impaired animals by (Grayson et al., 2007) as well as in the MRS chapter of this thesis. Line crossing data also showed no significant differences between both groups, demonstrating that there was no effect on locomotor activity in the Aβ treated group that could account for the selective cognitive deficits observed.

These findings in the NOR task suggest a strong cognitive decline in the rats on Day 14 following ICV administration of Aβ Oligomers. Studies show, following acute administration with Aβ oligomers (Desbene et al., 2012), as early as 3-4 days, the Aβ oligomer animals show a significant reduction in cognitive ability when compared to vehicle rats. These findings are also supported in both acute and chronic experimentation using Aβ oligomers (Bouter et al., 2013) as acute administration have previously shown observable working memory deficits in the Y-maze task. This study uses cDNA coding for $A\beta_{4-42}$ injected into fertilised C57BL/6J oocytes to cause chronic expression exposure of the Aβ oligomer. They also treated the rats with soluble $A\beta_{1-42}$ oligomers using an ICV injection acutely. 4 Days post ICV injection reductions in spatial memory in the water Morris maze was observed as well as CA1 pyramidal neuronal losses. This neuronal loss is caused by the chronic oligomer insult rather than the acute administration of the Aβ oligomers by causing a downstream pathologic process, including intracellular calcium dyshomeostasis, production of reactive oxygen species, altered signalling pathways, and

mitochondrial dysfunction that represent key effectors of cellular dysfunction and cell death (Glabe and Kayed, 2006). This could be due to the naturally secreted Aβ oligomers impairing synaptic function and causing a block on hippocampal long term potentiation (Haass and Selkoe, 2007). Since we used an acute administration method we can see the synaptic dysfunction. However, the oligomers will not, in our case, cause the block in the hippocampus. The findings shown in this paper suggests a long lasting behavioural deficit with memory dysfunction that can associate to AD. These findings were also replicated by (Youssef et al., 2008); however only using an acute dose of Aβ oligomers showing cognitive deficits in the Morris water maze and Y-maze. These findings suggest that behavioural deficits can occur after an acute load of the soluble Aβ oligomers and may be related to the preclinical stages of AD. The cognitive dysfunction seen in these experiments may be due to the oligomers action of selectively binding at membrane targets as ADDLs (high affinity ligand for synaptic terminals) (Lacor et al., 2004). This is believed to be as the ADDLs exerting pathogenicity on genes fundamentally important for the formation of long term memory. Another proposed theory of action can be that the Aβ oligomers cause multiple effects on the neurons through oxidative stress and/or neuro-inflammation that can lead to synaptic damage. In subsequent studies in the lab we have demonstrated that the deficits in NOR, following acute Aβ administration, are still present 70 days post-administration and that these deficits are accompanied by changes in both pre- (SNAP250 and post- (PSD95) synaptic markers (William Watremez, 2014).

Longitudinal behavioural studies in the transgenic model of AD show relevant results into the progression of the disease. Work done by (Havas et al., 2011) using the transgenic mouse model over-expressing the amyloid-β protein precursor using London (V717I) and Swedish (K670M/N671L) mutations in the presence of mThy-1 promoter (mThy1-hAβPP751) were analysed at 3, 6 and 9 months of age. The mThy1-hAβPP751 mice displayed an increasing number of Aβ plaques and mean plaque size in the cortex and hippocampus from 3 – 6 and 6 – 9 months of age indicating that changes can be observed in the long term stages of the disease and could be utilised for different drug testing time-points. This study also presented behavioural data also at 3, 6 and 9 months showing an age associated impairment in the learning ability in the mice in the water maze and hole-board tasks. Further work using a trans-genetic model of the AβPPswe, Tg2576 mouse model showed varying effects at 1, 3, 6 and 11 months of age (Lalande et al., 2014) using metabolic extracts of the frontal cortex, rhinal cortex, hippocampus, midbrain and cerebellum. At one month of age a decrease in NAA and glutamine was observed suggesting the time point at where a detectable change in NAA is observable using MRS on the hippocampus metabolic sample. Larger decreases seen again in the hippocampus for NAA and glutamine at three months, progressing to eleven months of age, where most brain regions were seen to be altered. The aforementioned findings show that an NAA deficit is more prominent in the later stages of AD progression. These findings differ from ours since a transgenic model focuses on a chronic model of Alzheimer's disease.

In our lab (and in partnership with SynAging) it has been hypothesised that early stage Alzheimer's disease memory loss has been correlated with synapse failure (William Watremez, 2014). This suggests that acute administration of Aβ oligomers will cause damage at the synapses, leading to the observed behavioural deficit. This however is based on the interpretation of previous results and needs further research to substantiate. If however the Aβ oligomers were given chronically via an osmotic mini pump and fibrils were able to form then we could replicate the reductions in NAA as shown with the genetic models. This perhaps suggests a further area of research that this work can progress into NAA deficits in AD.

Levels of NAA showed no significant difference between the Vehicle and Aβ oligomer animals in the brain regions investigated. There was a slight decrease in the levels of NAA in the frontal cortex, dorsal and ventral hippocampus of Aβ (1163.32±45.18 ng NAA/mg tissue, 791.28±106.88 ng NAA/mg tissue, 1014.04±28.75 ng NAA/mg tissue) compared to the respective vehicle treated controls (1172.12±34.37 ng NAA/mg tissue, 830.06±32.90 ng NAA/mg tissue, 1045.84±19.25 ng NAA/mg tissue) however not significantly different. This leads us to the belief that detectable neurodegeneration, as assessed with NAA, does not place take following acute Aβ oligomer administration. These small reductions suggest that earlier reports of a reduction in NAA levels in patient cortical grey matter in comparison with control groups (Adalsteinsson et al., 2000; Bittner et al., 2013; Frederick et al., 2004; Jessen et al., 2009) could be replicated with a chronic dose of Aβ oligomers. It is therefore possible to theorise that neuronal loss will occur in the later stages of the disease model (Buskila et al., 2013; Selkoe, 2002).

This is supported by the work of (Buskila et al., 2013; Selkoe, 2002) that there are subtle changes in the hippocampus that occur before neuronal loss and decline in the patients cognitive functioning which is reflected in our NOR experimental results.

The work done in this chapter displays some experimental similarities with the work published by (Nilsen et al., 2012). His work reports changes in metabolite levels in different brain regions in the early stages of the study however using MRS scanning and the R-Thy1-APP Alzheimer model. Nilsen's group found a decrease of NAA levels at 9 months however an increase in NAA at 12 months when compared to the frontal cortex in controls. However, there were lower NAA levels in the dorsal hippocampus at 3, 9 and 12 months with significance at 9 months.

In the work in this thesis the NAA levels in the Prefrontal cortex, striatum and temporal cortex (1061.9±43.52 ng NAA/mg tissue, 916.49±65.10 ng NAA/mg tissue, 1192.26±39.69 ng NAA/mg tissue) for the Aβ treated rats were higher, but not significant, to control animals (910.52±111.92 ng NAA/mg tissue, 910.52±18.44 ng NAA/mg tissue, 1011.78±89.62 ng NAA/mg tissue).

These findings again showed that neurodegeneration in this model did not occur in their experimental period. This could support the lack of NAA loss in our experimentation and the theory that only synaptic changes occur with an acute administration of the Aβ oligomers which is a powerful representation of early stage

AD. Findings shown as above lend support to the use of NAA as a biomarker in transgenic models for disease progression via neuronal dysfunction or death. A potential translational use of NAA is to highlight a patient's condition and disease stage and differentiate between patients with MCI and cognitively "normal" patients (Pilatus et al., 2009; Zhang et al., 2009).

Even though there were no NAA changes shown in these results there is an effect synaptically induced by the Aβ oligomers. In previous work in our group (W) following Aβ oligomer administration on day 35 there was a significant impairment in synaptic markers in the prefrontal cortex and hippocampus. Observed was a significant reduction in PSD-95 ($P<0.01$) in the prefrontal cortex as well as significant reductions of SNAP-25 in the prefrontal cortex and ventral hippocampus ($P<0.01$, $P<0.05$) with an increase in the dorsal hippocampus ($P<0.05$). In the specific areas being related to memory and cognition it shows relevant data to explain our findings in the NOR. The decrease in SNAP-25 supports our theory that there is synaptosomal damage that occurs with acute Aβ administration.

Neuro-inflammation can be measured by the biomarkers interlukin-1β (Shaftel et al., 2008) and caspase-1. These markers could also be used in a chronic administration of Aβ model using different time points to show the progression of action of the Aβ oligomers both cognitively and the pathology. As neuronal loss in the hippocampus is observed early on in AD another approach would be to use intra-hippocampal injection of the Aβ oligomers (Limon et al., 2012) to observe if a greater deficit can

be produced. Alternatively, a micro osmotic pump could deliver the Aβ oligomers into the brain in a specific region of interest for a prolonged period which would show if an accumulation of Aβ oligomers had a different effect on the brain (Lecanu and Papadopoulos, 2013). Transgenic animals have been shown previously to represent chronic Alzheimer's disease using a TgF344-AD line rat model to express the human amyloid precursor protein (Cohen et al., 2013). In older (24 month) TgF344-AD rats, a significant impairment in NOR data was seen as well as in the Barnes maze. This was accompanied with cerebral amyloidosis, gliosis and apoptotic loss of neuronal cells in the hippocampus and cerebral cortex using immunohistochemistry analysis. This publication shows a highly translational chronic model that has had previous success in showing neurodegeneration that could be adapted to use NAA as a biomarker for neuronal dysfunctioning. One of the main limitations of such an approach is the timescale in which the studies could be conducted and the relevance of transgenic models to sporadic AD.

As a comparison to the findings in this chapter a study done in patients with Alzheimer's disease showed direct correlations between NAA levels and Alzheimer's disease Cooperative Study Activities of Daily Living (ADCS-ADL) scores (Gordon et al., 2012). When comparing AD patients (mild-moderate) to controls there was a significant reduction in NAA in the precuneus and posterior cingulate regions. This also showed a direct correlation to the ADCS-ADL scores the patients received showing that IQ and disease progression can be measured by NAA in AD as shown by associations in the literature. We however did not see the correlations mentioned in our experimentation in relation to NAA levels. This is

theoretically due our acute administration of the Aβ oligomers. As mentioned before acute administration has, hypothetically, a short lived effect on the neuron synapse. Further chronic administration experimentation is required further to understand the ability of NAA to be used as a biomarker. Similar results were seen in the work by (Ackl et al., 2005) as a marked reduction in the NAA/Cr ratio was seen in the hippocampus of both AD patients and patients with mild cognitive impairment (MCI, group with elevated risk of developing AD). This suggests that our model may reflect an early stage MCI model at the time point we tested at, rather than a fully developed AD model. NAA in AD patients has been shown to be able to display improvement compared to drug interventions (Henigsberg et al., 2011). NAA in the dorsolateral pre-frontal cortex was shown to increase in relation to a higher treatment response with Donepezil again supporting the use of NAA as a biomarker for testing novel compounds and their efficacy.

The findings in this chapter as a whole show that a combination of the measurement of NAA and NOR experiments can be used as a model for testing novel therapeutic pharmacological compounds for the improvement of cognitive functions in AD. A significant reduction in the cognitive ability was shown in the NOR experiment in the Aβ oligomer treated rats, whereas the levels of NAA, in this chapter, displayed no significant increase or decrease when compared to controls animals. Our findings show association with early disease effects in Alzheimer's disease lending support to the results shown in this chapter.

With our results and the findings in current literature it is clear that further work is needed to better support NAA as a strong biomarker in AD. This could be achieved in a similar Aβ oligomer model but using the MRS methodology previously mentioned in this thesis. This would allow both behavioural testing and MRS scans to be completed over an acute or longitudinal study (1-11 months at set scan points as previously mentioned in the literature) with confirmation HPLC determination of NAA levels after the end of the experimental procedure with the use of an osmotic mini pump to administer constant soluble Aβ oligomers. The findings in this chapter suggest that acute ICV administration of soluble Aβ oligomers causes a robust cognitive deficit at 14 days but show no neuronal loss, as a reduction in NAA was not observed. With the results seen in human studies, and the comparison to this study, it shows promise in the use of NAA as a biomarker for assessing Alzheimer's disease progression and neurodegeneration.

Chapter 6

General Discussion

6.1 General Discussion

Findings in this thesis have shown that NAA has a promising ability to be used as a translational biomarker in neurological diseases in pre-clinical models which have been mirrored in human post mortem tissue. Although NAA has been used as a marker in a wide selection of publications and literature, there has been little overview of this useful molecule of its potential to help the development of new target compounds. It is widely accepted that NAA is a marker of neuronal dysfunction (Clark, 1998). However, there is fragmentation in the literature of the underlying role of NAA and its functioning in the brain (Coyle, 2006). Therefore, further research into the underlying mechanism of NAA is paramount. However, recently NAA is gaining interest as a biomarker and is becoming more widely studied (Harte et al., 2005; Harte et al., 2004; Nudmamud et al., 2003a; Reynolds and Harte, 2007; Reynolds et al., 2005; Reynolds and Reynolds, 2011; Xi et al., 2011) and hopefully the full potential of this molecule will soon be realised by the wider research community. It is from this previous research that this thesis takes its basis and provides a further stepping stone for NAAs research development.

While the findings in this thesis show promising results that can be further developed, there are a much larger assortment of neurological diseases that were not covered in this thesis that could be analysed, in greater detail, by NAA for a better understanding (Schuff et al., 2006). Although the diversity of diseases in this thesis is limited, the techniques that have been used show that there is promise in further research. The main power of NAA as a biomarker comes from its ability to highlight the difference in a reversal in the pathology of the disease in comparison to a short

lived pharmacological effect that most novel compounds present today. As well as the marked consistency of the data values of NAA in both *in vivo* with behavioural experimentation in comparison to *ex vivo* testing.

Lister-hooded rats were chosen for the *ex vivo* experiments in this thesis due to the previous success with this strain in our group (Neill et al., 2010) as they have been shown to have a higher level of performance in cognitive testing. The deficits are also shown to be robust in previous experiments and therefore were the natural choice as experimental subjects in the chapters for this thesis.

Before the main experiments were performed a large amount preliminary work had to be done to establish that the levels of NAA could be observed using the HPLC methodology. Although the work was based on previous studies (Harte et al., 2005), due to equipment limitation (SAX extraction columns), we had to modify the methodologies and develop new strategies for the extraction of NAA from neuronal tissue. This work required the largest amount of time to develop before the main experiments could occur. These preliminary studies were invaluable to show where the experiments could fail or if the proposed methods proved too complicated or the equipment issues would arise (Festing, 2004). This required different exchange columns to be tested to try and provide the highest retention percentage.

Preliminary experimentation was also carried out for the MRS scanning by scanning a rat before the main experiment took place to ensure that the results produced were

accurate. To do this we took a control rat from another cohort of animals, then scanned the rat on the MRS using the protocol listed in chapter 2. This allowed us to confirm the levels of NAA in the frontal cortex were measurable with the instrumentation we had, as well as refine the experiment methodologies. Following preliminary scan we found a clear, measurable NAA peak and therefore proceeded onto the main cohort of experimental animals for scanning.

The experiments in chapter 2 of this thesis were vital to show that NAA can be used to show a marked neuronal decrease in PCP treated animals. It was important to show where the marked decreases are located in the rat brain so as to target the further experiments in the rest of the chapters. The experiments show that there were significant deficits located in the frontal cortex as well as the ventral hippocampus. These findings allow us to better design the further experiments undertaken in both chapter 2 and in the rest of the thesis. It is from these findings that the main hypothesis that neuronal dysfunction, caused by pharmacological (sub-chronic PCP i.p. 7 day dosing / Aβ oligomers acute administration) or disease insult (schizophrenic brain samples), can show a marked decrease in NAA levels. In support of this data a reduction in the number of neurons or glial cells was shown in the PCP treated animals, further validating the findings shown in this chapter. This decrease in NAA was not reversed following chronic administration of the atypical antipsychotic olanzapine, demonstrating that its beneficial effects on symptoms are due to its pharmacological effects on different receptors and it does not affect the underlying pathology. This chapter shows the underlying theme for the rest of the thesis.

Chapter 3 was the next logical step in showing NAA's ability to be used as a neuronal biomarker, to move from *ex vivo* analysis and determine if we could replicate these results *in vivo*. As MRS scanning uses NAA in a large amount of literature, it was a sensible methodology to apply to this. The frontal cortex was chosen to be the focus of the scan due to the significant reductions reported in chapter 2. Following post scan analysis of the data we again found a significant reduction in NAA levels in the frontal cortex, showing similarity as to what we found in chapter 2. To support the findings, an NOR test was chosen to be carried out at the end of the scanning period. An NOR with a short ITI (1 min) was chosen to tailor the region of recognition memory at the frontal cortex (Cohen and Stackman Jr, 2014; Hammond et al., 2004). NOR testing showed a deficit in the PCP treated animals compared to vehicle in the retention phase therefore supporting the NAA findings in this and the previous chapter. Following the *in vivo* scanning and behavioural testing, brains were taken and analysed for NAA using HPLC. Interestingly we found a similar deficit in NAA levels in the PCP treated group. This study provided, for the first time, a clear indication of the association between *in vivo* (MRS) and *ex vivo* (HPLC) analysis in the same tissue.

Human brain samples from the Stanley Foundation were used to look at levels of NAA in the frontal cortex in control and schizophrenics. Patient demographics showed no significance between groups for age, gender, post mortem interval (PMI), brain hemisphere or ethnicity. In this study brain tissue was separated into grey and white matter as to discern if there was an NAA reduction in neuronal cell bodies and/or axons. Significant reductions were seen in the grey matter in the schizophrenic patients when compared to controls after using the aforementioned

HPLC method of analysis. This reduction, however, was not seen in the white matter of the patient groups. Statistical analysis demonstrated no correlation between NAA levels and brain PH, brain weight, age, refrigeration interval, PMI, age of onset, duration and lifetime antipsychotics. We found there was a statistical correlation of PMI in the white matter. This is believed to occur as NAA levels will degrade the longer the time interval from patient death to freezing of the brain samples. However, we did not see this correlation in the grey matter. This could be due to a higher overall NAA level masking a small decrease in post mortem NAA levels. Since this does not affect the grey matter we can safely rely on our findings in this experiment. The findings shown in chapter 4 show a direct association between NAA levels and disease status of the patient, including the previous chapter's work, we can safely draw to the conclusion that a reduction in levels of NAA are a strong marker of neuronal dysfunction.

In subsequent studies we investigated levels of NAA in a preclinical model of relevance to the early stages of Alzheimer's disease to create a diversified view of NAA levels in neurological diseases. In previous work, our group has developed the Aβ oligomer model of relevance to Alzheimer's disease that could effectively be used to measure NAA levels. We were successful in showing that there is a behavioural deficit (NOR) following an acute dose of the soluble Aβ oligomers. We showed that there was no significant change in the levels of NAA which led to the theory that an acute dose of Aβ only causes synaptosomal changes and not neuronal dysfunction. Even though we did not observe a significant decrease in NAA there was a small decrease in the frontal cortex, dorsal and ventral hippocampus showing that NAA is sensitive enough to show subtle changes in the neuronal functioning.

This finding suggests that NAA can be used to measure subtle neuronal changes. By measuring these NAA levels we can effectively observe what occurs in an early stage of Alzheimer's disease.

There is a constant increasing interest in NAA as a marker of neuronal dysfunction since NAA's discovery by Tallan in 1956 especially since the 1980s. The use of NAA as a biomarker is a simple, hi throughput experiment that after setup can provide powerful results, either with the use of HPLC (post mortem analysis) or through MRS analysis. With NAA being easily measured, and the second most abundant metabolite in the human brain, it is a perfect candidate for a translational biomarker. NAA has already been noted to be reduced in neurodevelopmental disorders, brain diseases and acute trauma (Barker, 2001). There has been previous mention of NAA being used as a neuronal marker but for neuronal loss or mitochondrial dysfunction (Clark, 1998), however, it is the dysfunction of normal neuronal function that was the focus of this thesis. There was support for our underlying hypothesis in that NAA has already shown a direct association with cognitive functioning (Ross and Sachdev, 2004) which was partially replicated in our results with the use of the NOR testing. It is these relationships between NAA and neuronal dysfunction that will prove clinically and preclinically useful as to developing our understanding of disease progression, as well as drug development or tailoring to patient needs. It is important to mention that a visible reduction of NAA can also represent neuronal death as well as dysfunction. However patients with traumatic brain injury show evidence of a reversible decrease in NAA levels accounting for reversible neuronal dysfunction (Brooks et al., 2001). It has been suggested that NAA has the ability to measure more than just neuronal

dysfunctioning and death. It was reported by (Ross and Sachdev, 2004) that NAA is also a measure of reductions in the area of dendritic arborisations as well as a reduction in myelination. Recent studies have also shown that an inhibition of the mitochondrial respiratory chain leads to a reduction in the levels of NAA produced, reflecting neuronal dysfunction on a sub cellular level (Bates et al., 1996). These findings show a positive future for NAA as it can be used to measure a wide variety of known neurological disease effects. It is, however, important to note that NAA in some cases may be interpreted as a marker for metabolic impairment rather than as a biomarker for diseases or neurodegeneration.

In most disease states one of the most common, yet debilitating factors, is the reduction in cognitive functioning, preventing patients from carrying out their day to day lives. As NAA can measure a variety of neuronal insults it will support the development of cognitive enhancing compounds. This is because NAA, being an indicator of dendritic arborisations, are related to information processing and capacity (Yeo et al., 2000). Fluctuations in neurons and glial cells (as shown in chapter 2) relate to a greater number of neurons or glial cells being related to a higher IQ or improved scores in cognitive function tests such as CANTAB (Scheibel et al., 1990). Increased conduction speed of neurons, due to larger neurons, has also been linked with a greater speed of processing which in turn would display increases in NAA levels (Jung et al., 1999b). In relation to neuronal transmission speed, an increase in myelin thickness would help to boost this. Since NAA has been seen to play a role in myelin synthesis due to it being a donor of putative acetyl groups an increase in NAA would be seen with an increase in myelin thickness (D'Adamo et al., 1968).

It is important to note that we managed to replicate the decrease in NAA in chapter 2 of this thesis using the HPLC with the MRS methodology in chapter 3. This ability to replicate the same findings in both the methodologies is vital to show that both techniques can show a reliable deficit as well as validating themselves. All these finding show support that NAA can be used as a diagnostic biomarker and will show reductions in abnormal brain states that can be shown both neurochemically as well as behaviourally.

6.2 Future work with NAA

This thesis shows how NAA can be used in an analytical setting and hopefully will lead to NAA being used as a more diagnostic role in the clinic as well as in pre-clinical development. NAA has the potential to improve the current understanding of drug disease interaction as well as disease development. There is a large amount of work still to be done with NAA. One of the most important is the discovery of a solid role of NAA in neuronal functioning that would greater develop our understanding of the neuronal NAA link. Another is NAA's location in the neuron. In our laboratory we began developing a method of measuring the level of NAA in synaptosomes to show the location, if any, of NAA reduction in a PCP model. With further work in this area we could develop the understanding of NAA. Work in the clinic setting, following cognitive remediation in schizophrenic patients, could be further improved by measuring the levels of NAA to see if there is a reversal in neuronal dysfunction.

In relation to the data in the Alzheimer's disease chapter, where no significant changes in NAA were found, further research is required to discover the ability of

NAA to be used as a biomarker. With the current results we can conclude that NAA lacks the ability to be a measure of neuronal dysfunction in an acute administration model using Aβ oligomers. Therefore, to expand this area of study, a chronic model may need to be implemented to identify the brain areas where NAA loss can be detected.

6.3 Limitations of the HPLC

Although the HPLC has a high accuracy of measurement accuracy of NAA, there are some limitations to the methodology. Before the samples can be run, the use of SAX extraction columns are needed to remove NAA from the rest of the brain matter. Even though a high amount of NAA is retained, there is still a small amount of loss of the molecule that has to be factored into the final calculations which could account for a small variation in the levels of NAA. This, however, is unavoidable but with more preliminary experiments, this loss can be reduced further.

We also found that prolonged use of the HPLC column caused a blockage of the system due to unavoidable contaminants in the samples. The meant that samples needed to be run on a fresh column in batches and that the system was left on constant running cycle to prevent build-up of the contaminants.

6.4 Future Studies of the HPLC

Further analysis of the effect on disease medications would provide an insight into the mechanism of action with the use of NAA to show if effects on pathology could be observed. A wider range of disease analysis could provide a greater breadth of understanding of NAA and how it is affected in disease states.

Despite us not finding a reversal in the levels of NAA following olanzapine administration, research has been carried out in this area, with other compounds, that have shown to have an effect on NAA levels (Jarskog et al., 2013). This, therefore, shows there is still promise for the use of NAA in the development of novel compounds. Therefore, the methodology used in this thesis could be adapted to be used in a high throughput screening technique for novel compounds to provide drug companies a better understanding of the mechanism of action on the pathology of diseases. The advantage to HPLC is that it is a low cost high data return which could provide invaluable insight to further the development of novel drugs in a neurological setting.

6.5 Limitations of the MRS

The MRS methodology is a tried and tested method for measuring levels of NAA and provides an accurate measure in an *in vivo* setting. We were limited on the time on the MRS due to it being communal use equipment and the schedule only allowed us to use n of 5 per group. The scanning method also presented lipid infiltration that

produced peaks on the MRS spectrum. Before analysis of the data the lipid infiltration had to be factored and removed, so as to not skew the data.

6.6 Future work with the MRS

As was mentioned in chapter 3, an increase in number would confirm our results in this experimentation. Since we have shown that the levels of NAA in an animal model can be measured, the next logical step would be to measure the NAA levels during and after administration of an atypical antipsychotic and/or novel cognitive enhancers in the sub-chronic PCP model. This would allow us to see the action of a particular compound as it acts *in vivo* as well as any long term effect on NAA levels. This methodology also shows potential of using a neurodevelopmental model to track changes at different time points in the same cohort of animals. This allows us to look at the onset of deficits and prevention strategies in neurodevelopmental diseases. This would provide invaluable information as to how compounds affect the neurochemistry of the brain. The recent work by (Jarskog et al., 2013) have used NAA in this way in a clinical setting. In their study, using schizophrenic patients, they received either placebo or davunetide (5 - 30 mg/day). Although there was no significant result found, there was a trend of NAA increase (8% - P=0.072) in the patients receiving the highest dose of davunetide when compared with placebo. The increase in NAA observed is theorised to be due to the neurotrophic action of davunetide, linked to the stabilization of microtubules and restoration of cognitive deficits. This is the first time that NAA has been used as a biomarker for assessing the effect of treatment on neuronal function. This is exactly what we hope to show, in this thesis; NAA is useful for in a clinical and preclinical setting.

6.7 Conclusions

The results shown in this thesis demonstrate the usefulness of NAA as a marker of neuronal dysfunction and its ability to show levels of decline in a diseased brain, both models and human samples. The preliminary work is invaluable to show that these levels can be measured easily and effectively with a high degree of accuracy. Initial analysis shows that conditions of the experiment must be calibrated to high NAA retention. Clearly, if this had not been carried out, the results of all following experiments could have been skewed. Initial experimentation shows that NAA levels can be measured in a model of schizophrenia *in vivo* and *ex vivo* in animal models. Following the initial experiments we can replicate these findings not only in human samples of schizophrenic patients but in a model of Alzheimer's disease. With all of the following findings, supported by behavioural work, we can rely on the findings shown.

In summary, NAA can successfully be used to measure neuronal or metabolic dysfunction in a wide variety of animal models as well as in humans showing high translational ability of this biomarker. With the use of HPLC and MRS detection methods drug efficacy on the pathology in diseases can be easily and effectively measured in a repeatable and quantifiable methodology. Further study is required to refine the NAA biomarker, as well as cell localisation and mechanism of action in neurons to further develop understanding of this invaluable biomarker.

Appendix

Appendix A

Protocols and Standards Lookup Sheet

HPLC Solutions & Column Preparation:

- 0.1 M Perchloric Acid (PCA)
- 0.2 M Sodium Carbonate
- 1 M Phosphate Buffer pH 6
- 50 mM Phosphate Buffer pH 6
- 2 mM Phosphate Buffer pH 6
- 0.1% Phosphoric Acid (PhosA)
- 0.5% Phosphoric Acid
- 1.5 M Sodium Hydroxide

0.5% Phosphoric Acid, H_3PO_4

Have a stock of 85% PhosA.

2.95 ml PhosA into 500 ml H_2O = 0.5%

2.36 ml PhosA into 2 L H_2O = 0.1% (MOBILE PHASE FOR NAA & NAAG)

0.2 M Sodium Carbonate, H_3PO_4, 105.99 g/mol

105.99 g into 1 L H_2O = 1 M

1.0599 g into 10 ml H_2O = 1 M

0.212 g into 10 ml H_2O = 0.2 M

0.1 M Perchloric Acid, $HClO_4$

100.46 g into 1000 ml H_2O = 1 M

10.046 g into1000 ml H_2O = 0.1 M

5.023 g into 500 ml H_2O = 0.1 M

7.195 ml into 500 ml H_2O = 0.1 M

1.5 M Sodium Hydroxide, HNaO

$40 gmol^{-1}$

60 g Sodium Hydroxide into 1000 ml H_2O = 1.5 M

Phosphate Buffer

NaH_2PO_4 137.99 $gmol^{-1}$
Na_2HPO_4 141.96 $gmol^{-1}$

All are made to pH 6:

1 M
103.5 g NaH_2PO_4 into 750 ml
35.49 g Na_2HPO_4 into 250 ml

50 mM
3.45 g NaH_2PO_4 into 500 ml
1.77 g Na_2HPO_4 into 250 ml

2 mM
0.138 g NaH_2PO_4 into 500 ml
0.069 g Na_2HPO_4 into 250 ml

HPLC Standards

NAA

175.1 g into 1000 ml H_2O = 1 M
1.751 g into 10 ml H_2O = 1 M
1.751 mg into 10 ml H_2O = 1 mM
17.51 mg into 10 ml H_2O = 10 mM

*= Standard solution for storage and then usage in the HPLC
Split all 10 ml into 1 ml aliquots. Store 1ml for use in the fridge and the rest in -20^oC freezers.

NAA for Un extracted Standards:
Dilute 100 µl [17.51 mg into 10 ml H_2O = 10 mM] dilution with 900 µl 50 mM phosphate buffer = Solution B (1 mM NAA)

10 µl Solution B + 1490 µl 0.5% Phosphoric acid
30 µl Solution B + 1470 µl 0.5% Phosphoric acid
100 µl Solution B + 1400 µl 0.5% Phosphoric acid

NAA for Extracted Standards for Column Preparation:
Dilute 100 µl [17.51 mg into 10 ml H_2O = 10 mM] dilution with 900 µl 50 mM phosphate buffer = Solution B (1 mM NAA)

10 µl Solution B + 990 µl 50 mM Phosphate Buffer
30 µl Solution B + 970 µl 50 mM Phosphate Buffer
100 µl Solution B + 900 µl 50 mM Phosphate Buffer

NAAG

When the solution comes it must be stored in the -20^{o}C freezer.
The solution must be made up with 1.5 ml EtOH to aid dilution.

304.3 g into 1000 ml H_2O = 1 M
3.043 g into 10 ml H_2O = 1 M
30.43 mg into 10 ml H_2O = 10 mM
3.043 mg into 10 ml H_2O = 1 mM

NAAG for Un extracted Standards:
Make up a 1:1 dilution of 1mM NAAG with 10mM Phosphate buffer (Solution C – 0.5 mM NAAG)
Make up a 1:1 dilution of Solution B with 10mM Phosphate buffer (Solution D – 0.25 mM NAAG)

10 µl 1 mM NAAG + 1490 µl 0.5% Phosphoric acid
10 µl Solution C + 1490 µl 0.5% Phosphoric acid
10 µl Solution D + 1490 µl 0.5% Phosphoric acid

Preparation of Sample Extraction Columns

1ml Vol, SAX, Ion exchange, 100 mg/1 ml

Run through the columns in the following order:
[Run all to waste]

2. 1 x 1 ml Methanol
3. 2 x 1 ml 0.5% Phosphoric Acid
4. 4 x 1 ml 1 M Phosphate Buffer
5. 2 x 1 ml 50 mM Phosphate Buffer

6. Add sample (1 ml) and run to waste

7. 2 x 1 ml 2 mM Phosphate Buffer
8. 1 x 1 ml Ultra-pure H_2O

[COLLECT IN 1.5 ml EPPENDORFS]

9. Elute samples with 1.5 ml 0.5% Phosphoric Acid

Preparing Tissue Homogenisation

Used for collection of white and grey matter of human samples.

1. Scrape relevant tissue sample into Eppendorfs – Separate for White and Grey matter
2. Add 61 µl, 0.1 M Perchloric Acid to Eppendorfs
3. Vortex
4. Place on ice for 5 minutes
5. Vortex
6. Spin at 12,000 rpm for 5 minutes
7. Remove supernatant, 50 µl and place into an Eppendorf with 30 µl Na_2CO_3 0.2 M and 920 µl 50 mM Phosphate buffer. (This is the sample for the extraction column procedure)
8. Freeze pellet

Protein Assay

Taking Brains

1. Take brain from -80ºC freezer and let slightly thaw
2. Chop into 10 and place onto dry ice
3. Weigh tissue into a new eppendorph and place onto normal ice
4. Add 10x volume of Perchloric acid 0.1 M. e.g. 40 mg = 400 µl
5. Homogenise then place on ice then re homogenise until it forms a cloudy liquid.
6. Spin at 12,000 rpm for 5 minutes
7. Remove supernatant and freeze
8. Use pellet for protein assay

Protein Assay

1. Make 1.5 M Sodium Hydroxide
2. Make Bradford Reagent:
    a. Place 1 mg Coomassie Blue – G into 0.5 ml EtOH
    b. Add 2 ml of 85% Phosphoric Acid
    c. Make up to 10 ml with pure H_2O
    d. Colour of solution should be brown/red
3. Make Bovine serum albumin (BSA) 2 mg/ml in sodium hydroxide 1 M
4. Dissolve protein pellet with 1 ml of 1.5 M sodium hydroxide.
5. Make a 1:5, 1:10 and 1:20 dilution in triplicate of each sample from the pellet and always run one pure sample, again in triplicate.
6. Prepare a serial dilution of the BSA, 1, 0.5, 0.25, 0.0625 & 0 mg/ml.
7. Place all BSA and samples in triplicate into the 96 well plates in a 10 µl volume.
8. Add 300 µl of the Bradford reagent to all the wells
9. Read the plates at 595 nm

10. Plot a standard curve of the average of the BSA results.
11. Check that the curves $R^2>0.95$
12. Read the equation of the trend line and rearrange (read from right to left)
    a. y=ax+b -> (y-b)/a=x
13. Fill in values and produce data using the equation and the averages of the samples results.

Appendix B

TAC CODE	Control3/Schiz4	DSMIVAGE	Sex	Race	RefInt	PMI	BrainPH	LeftBrain	RightBrain	BrainWgh	AgeofOnset	Duration	LifetimeAntipsy	Left or Right
12	Control	31	1	1	3	11	6	2	1	1335		0	0	1
17	Control	51	1	1	7	22	7	1	2	1900			0	2
28	Control	50	1	1	6	49	7	2	1	1645			0	1
29	Control	48	1	1	3	31	7	2	1	1580			0	1
48	Control	45	1	1	2	18	7	1	2	1585			0	2
49	Control	57	1	1	0	26	6	2	1	1470			0	1
52	Control	44	2	1		10	6	2	1	1305			0	1
65	Control	35	1	1	3	52	7	2	1	1700			0	1
70	Control	60	1	1	4	47	7	2	1	1460			0	1
73	Control	38	2	1	3	28	7	2	1	1350			0	1
75	Control	38	2	1	3	33	6	2	1	1120			0	1
77	Control	53	1	1	2	28	6	1	2	1340			0	2
78	Control	51	1	1	2	31	7	2	1	1400			0	1
79	Control	37	1	1	2	13	7	1	2	1600			0	2
82	Control	53	1	1	2	9	6	1	2	1500			0	2
13	Schiz	295	42	1	1	3	19	6	2	1	1310	18	24	18000	1
14	Schiz	295	47	2	1	10	35	7	1	2	1575	20	27	90000	2
16	Schiz	296	42	1	1	2	26	6	2	1	1410	24	18	10000	1
27	Schiz	296	47	2	1	3	30	6	2	1	1430	23	24	15000	1
53	Schiz	296	47	1	1	1	13	6	1	2	1310	20	27	30000	2
64	Schiz	296	32	2	1	5	36	7	1	2	1340	29	3	10000	2
67	Schiz	296	43	1	1	2	18	6	2	1	1520	18	25	90000	1
68	Schiz	295	50	1	1	1	9	6	2	1	1400	31	19	34000	1
69	Schiz	296	33	1	1	5	29	7	1	2	1470	19	14	20000	2
71	Schiz	295	39	1	1	8	80	7	1	2	1355	17	22	120000	2
76	Schiz	296	44	1	1	4	9	6	1		1415	19	25	350000	2
80	Schiz	296	24	1	1	5	15	6	2	1	1505	20	4	12000	1
81	Schiz	295	52	1	1	2	10	6	2	1	1450	28	24	10000	1
94	Schiz	296	19	1	1	11	28	7	1	2	1465	18	1	2500	2
95	Schiz	296	51	1	1	4	43	7	1	2	1390	23	28	130000	2
96	Schiz	296	40	1	1	2	34	6	2	1	1480	21	19	75000	1
99	Schiz	295	31	1	1	7	33	6	2	1	1480	22	9	35000	1

Table 18: Human Patient Demographics

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