MAGNETIC RESONANCE SPECTROSCOPY (MRS) TO DOCUMENT CHANGES IN NEUROCHEMISTRY.

Scott Quadrelli MBBS; B.Eng(Hons 1)

A thesis submitted in fulfillment of the requirements for the degree of Doctor of Philosophy in Magnetic Resonance in Medicine.

July 2018

School of Health Sciences

Faculty of Health and Medicine

University of Newcastle

This research was supported by an Australian Government Research Training Program (RTP) Scholarship

Statement of Originality

I hereby certify that the work embodies in the thesis is my own work, conducted under normal supervision. The thesis contains no material which has been accepted for the award of any other degree or diploma in any University or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. I give consent to the final version of my thesis being made available worldwide when deposited in the University's Digital Repository, subject to the provisions of the Copyright Act 1968.

Thesis by Publication

I hereby certify that this thesis is in the form of a series of published papers of which I am a joint author. I have included as part of the thesis a written statement from each co-author, endorsed by Assistant Dean of Research Training, attesting to contribution to the joint publications (as shown in the Appendix).

Dr Scott Quadrelli

Acknowledgments

I dedicate this thesis to my daughters, Isobel and Mia.

My PhD has been a challenging and a rewarding experience, thank you to all those who have helped along the way.

Firstly, I want to thank my supervisors, specifically Carolyn Mountford and Saad Ramadan who have taught me in an enormous amount during the course of this PhD, in both the technical aspects of MRI spectroscopy and how to be an effective academic. A special acknowledgment goes to Professor Mountford, who spoke at John Hunter Hospital at the time of my residency, the talk was on MRI spectroscopy. If it wasn't for that lecture I would not have started this research and would not have gone on to commence postgraduate training in Radiology, a field which appears to be a perfect fit for me. I also wanted to thank Karen Ribbons and Jeanette Lechner-Scott, for all their help and guidance with the multiple sclerosis component of this thesis. Thank-you Jameen Arm, for all your help in the early days at HMRI.

I would like to thank my family, both past and present, without the support and sacrifices of whom, I would not be doing what I am able to do today. Mum and Dad, thank you so much for all of the opportunities and encouragement that you have given throughout the years.

Lastly and most importantly, thank you to the love of my life, Lauren. The sacrifices that you have made, has made this PhD possible.

Publications arising from this thesis

Chapter 2 – Published.

Mountford, C., **S. Quadrelli**, A. Lin and S. Ramadan (2015). "Six fucose-alpha(1-2) sugars and alpha-fucose assigned in the human brain using in vivo two-dimensional MRS." NMR in Biomedicine **28**(3): 291-296.

Chapter 3 – Published.

Quadrelli, S., C. Mountford and S. Ramadan (2016). "Hitchhiker's Guide to Voxel Segmentation for Partial Volume Correction of In Vivo Magnetic Resonance Spectroscopy." Magnetic Resonance Insights **9**: 1-8.

Chapter 4 – Accepted.

Quadrelli, S., C. Mountford and S. Ramadan (2018). "Systematic review of *in-vivo* neuro Magnetic Resonance Spectroscopy for the assessment of Posttraumatic Stress Disorder" Psychiatric Research: Neuroimaging.

Chapter 4 – Submitted and under review.

Quadrelli, S., N. Tosh, A. Urquhart, K. Tricky, R. Tremewan, G. Galloway, L. Rich, R. Lea, P. Malycha, and C. Mountford. "Posttraumatic Stress Disorder Affects Fucose-α(1–2)-glycans in the Human Brain: Preliminary Findings of Neuro Deregulation using In Vivo Two Dimensional Neuro MR Spectroscopy" Translational Psychiatry. Submitted 15/5/18.

Chapter 5 – Submitted

Quadrelli, S., K. Ribbons, J.Arm, O. Al-iedani, , J. Lechner-Scott, R. Lea and S. Ramadan (2018). "2D *in-vivo* L-COSY spectroscopy identifies neurometabolite alterations in multiple sclerosis" Radiology.

Abstract

Magnetic resonance spectroscopy (MRS) is a non-invasive technique that can be used to determine the chemical composition of biological tissues in a conventional MRI scanner. Due to its non-invasive nature, *in vivo* proton MRS has been referred to as a 'virtual biopsy' and allows a unique metabolic fingerprint of certain pathologies to be determined. There are a variety of technologies available to perform MRS, including one-dimensional (1D) and two-dimensional (2D) spectroscopy. 1D spectroscopy has several limitations, including difficulty separating peaks secondary to peak overlap. In contrast, 2D spectroscopy separates the resonances in a second magnetic frequency, allowing unambiguous assignment of metabolites and new molecules to be assigned that were previously not visible in the 1D spectrum.

The first contribution to arise from this thesis was to apply the novel spectroscopy technique, 2D MRS, specifically 2D-Localised COrrelated SpectroscopY (2D L-COSY), to assign and identify fucosylated glycans in the brain *in vivo*. Using 2D L-COSY up to six fucose-α (1-2) – galactose species were able to be successfully assigned. These species have previously been shown to be important in learning and memory. This is the first time in decades that a new metabolic assignment has been made in the brain *in vivo* using MRS.

Post processing is an important step in spectroscopy data analysis, ensuring accurate and reproducible results. In the second contribution to arise from the thesis, methods to perform partial volume correction for spectroscopy studies were developed and evaluated. Additionally, methods to extract metrics of interest, such as the number of white matter lesions, from a MRS voxel were determined and published. This allowed these metrics of interest to be correlated with the metabolic differences found using spectroscopy. These techniques were then applied in some of the clinical studies described below.

In this thesis, 2D L-COSY was applied to several clinical conditions, specifically Posttraumatic Stress Disorder (PTSD) and Multiple Sclerosis (MS) as well as the healthy brain. PTSD is a debilitating trauma and stressor related disorder that results in complex somatic, cognitive, affective and behavioural effects after an individual is exposed to a traumatic event. In the process of the study, a systematic review of the literature characterising metabolite differences in PTSD, assessed using MRS, was undertaken. Currently, there is no objective imaging diagnostic tool for PTSD, and there is a need for further imaging tools to diagnose and monitor the condition. Unlike PTSD, MS is a chronic autoimmune demyelinating disease that has typical imaging findings on MRI. Despite this, there is a disconnect

between the MRI imaging findings and the patient's clinical disease severity. There is a great need for further imaging bio-markers in MS. Given the emergence of multiple new biological disease modifying agents, further imaging tools are needed to determine the efficacy of new treatments.

Using 1D MRS and 2D L-COSY in PTSD, multiple metabolic differences were identified that separated patients from healthy controls. Using 1D spectroscopy, there was a reduction in absolute inositol, inositol: Cr in the posterior cingulate cortex, and an increase in (Glu): tCr and Glx: tCr in the posterior cingulate cortex. Reduced inositol in the posterior cingulate cortex has not been previously described using 1D MRS and may be secondary to apoptosis of astrocytes. Using 2D MRS additional metabolic differences in PTSD were identified, not previously described using 1D MRS, such as a reduction in total fucose and the fucosylated glycans fuc IV and VI in the posterior cingulate cortex. Fucosylated glycans are thought to be contained within synapsin proteins in the brain, and this may be the first *in vivo* evidence of dysregulation of synapsin in PTSD. The only metabolite (IMI-1) that correlated with clinical symptoms was found using 2D L-COSY.

Multiple differences in chemical signatures were again found using 2D L-COSY to quantify neurochemical changes in the brains of patients suffering from relapsing and remitting multiple sclerosis (RRMS). Specifically, a significant reduction in multiple N-acetylaspartate (NAA) signatures, GABA and Glx, was identified in the posterior cingulate cortex in RRMS when compared to healthy controls. Of the clinical symptoms measured, visual spatial function and attention were most correlated with metabolites in the brain. Here the first *in vivo* evidence has been provided that 2D L-COSY has the potential to detect metabolic alterations in the normal appearing brain in multiple sclerosis and PTSD. Metabolic variability associated with clinical symptoms was detected despite only examining a localised region in both conditions.

This research has shown that 2D L-COSY is a useful additional spectroscopy technique, which can be used to identify additional neurochemical changes in the brain, when compared to conventional 1D spectroscopy. Although technological advances are required, this technique may one day provide clinicians with much needed imaging biomarkers for conditions that have no conventional imaging or limited clinically relevant imaging findings.

Table of Contents

Chapter 1

General Introduction

Background

Magnetic resonance imaging (MRI) was pioneered by Lauterbur in 1973 using nuclear magnetic resonance (NMR) techniques. MRI creates images of objects based on their water content. This resulted in the development of wide bore horizontal magnets, used for human MRI scanning. However, MRI scanners can be used as a NMR spectrometer to acquire non-invasive data on the chemical composition of biological tissues. NMR has been an essential tool for organic chemistry, chemistry and biochemistry to aid in the determination of chemical structure and its relationship to pathology (Mountford, Stanwell et al. 2010).

Due to its non-invasive nature, *in vivo* proton Magnetic Resonance Spectroscopy (MRS) has been referred to as a 'virtual biopsy' and has allowed for a unique 'fingerprint' of certain pathologies to be characterised (Mountford, Stanwell et al. 2010), using a conventional MRI scanner at a field strength of 1.5 or 3T. In the brain, MRS has been applied to multiple different disease processes such as malignancy, Multiple sclerosis (MS), cognitive impairment and stroke (Oz, Alger et al. 2014). MRS can quantify parenchymal metabolism, in both healthy and diseased tissue and has the potential to provide biomarkers for psychiatric disease, which often has no characteristic findings on conventional imaging (Foerster et al., 2012; Murray et al., 2014; Ramadan et al., 2015). Up to 35 metabolites, lipids and macromolecule signatures can be quantified using *in vivo* one dimensional (1D) MRS in a 3T MRI scanner (Provencher 2001).

The majority of previous neurospectroscopy studies have utilised 1D spectroscopy. The main limitation of 1D spectroscopy is composite and overlapping peaks, of which there are many, that require deconvolution to accurately measure metabolite concentrations. Recent studies have employed 'edited' pulse sequences, such as MEGA-PRESS (Mescher, Merkle et al. 1998), allowing for greater selectivity in detecting a small number of metabolites such as gamma-aminobutyric acid (GABA), Glutamine (Gln) and Glutamate (Glu). Limitations of this technique include the low number of metabolites that can be quantified and long acquisition times.

2D Spectroscopy

Two-dimensional (2D) MRS is a technique that allows composite or overlapping resonances from a 1D spectrum to be separated in a second magnetic frequency. In conventional 1D spectroscopy, intensity (y-axis) is plotted against frequency (x-axis), whereas in 2D spectroscopy intensity is plotted against two frequency variables (Keeler 2010), allowing all metabolites to be quantified without the need for multiple pulse sequences.

2D spectroscopy was first introduced by Jeener and Goldman as a two pulse sequence known as correlated spectroscopy (COSY) (Ramadan, Ratai et al. 2010). The COSY pulse sequence comprises a 'preparation period' which consists of a 90° pulse, followed by an evolution period t_1, which is then followed by a 'mixing period' that consists of a second 90° pulse. The signal is then detected during the t_2 period. The time (t_1) of the evolution period is not fixed and is incremented gradually as separate experiments (Keeler 2010). In the first experiment, the free induction decay (FID) is collected after the t_1 is set to zero and the pulse sequence is executed as described above (Keeler 2010). During each experiment, the FID is collected in real time so collecting more t_2 data points does not increase the run time. The time of acquisition is however limited by the number of t_1 data points (Keeler 2010). The pulse sequence for COSY is shown below in Figure 1.

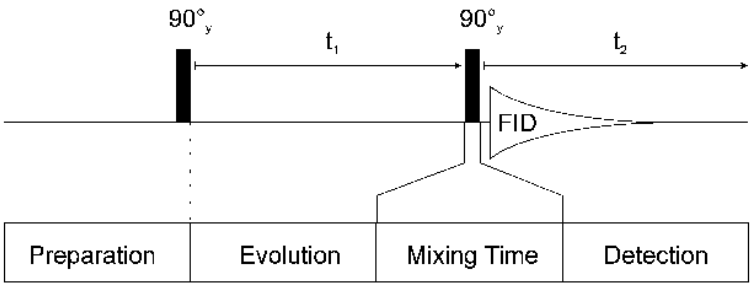


Figure 1 - COSY pulse sequence (source: http://www.cryst.bbk.ac.uk/PPS2/projects/schirra/images/2dexp_1.gif)

2D MRS spectroscopy found its way into the field of biology in 1984 when it was applied to cells and then later to tissue in 1988 (Cross, Holmes et al. 1984, Williams, Saunders et al. 1988). *In vivo* 2D spectroscopy was first undertaken in 1994 by Brereton, Galloway et al. at 2 Tesla, however the quality of the spectrum was poor due to the low field strengths (Brereton, Galloway et al. 1994). Sometime later, the *in vivo* technique was further developed by Thomas et al. (2001) and then Ramadan (2011).

The development of higher field strength MRI machines, as well as head coils with increasing numbers of channels has meant *in vivo* 2D spectroscopy can be developed with improved Signal-to-Noise Ratio (SNR) and spectral quality. Specifically, the introduction of scanners such as the Siemens 3T Prisma (Siemens, Erlangen, Germany) with a 64-channel head and neck coil has resulted in improved SNR and water suppression. To date, 2D L-COSY has been applied *in vivo* to breast malignancy and glioblastoma (Ramadan S, Andronesi O et al. 2011, Ramadan, Arm et al. 2015). Its usefulness is now examined for two diseases states, posttraumatic stress disorder and multiple sclerosis.

Thesis Rationale

Magnetic resonance imaging is a powerful technique that is used frequently in routine radiological practice, however, for many pathologies, such as MS and posttraumatic stress disorder (PTSD), there is a disconnect between imaging findings and the disease phenotype. In the case of PTSD there are no diagnostic imaging findings using conventional MRI and the neural mechanisms underlying the pathology remain unclear. MR spectroscopy has the potential to identify unique metabolic fingerprints, secondary to altered neuronal metabolism, and therefore imaging biomarkers, not available using conventional imaging.

This thesis initially focuses on the assignment of a series of fucosylated molecules in the brain as well as the substrate α-L-fucose. Additionally, the impact of partial volume correction and its impact on the postprocessing of spectroscopy data is explored and a method and code to deal with this problem is implemented and published. As part of this work, an automated method to extract metrics of interest from a MRS voxel is developed, such as the number of white matter plaques in MS, allowing correlations to be made between imaging and MRS metrics.

Having made the Fucose assignments and developed automated techniques to deal with partial volume correction, 2D L-COSY is applied to two very different pathologies, PTSD and MS, two conditions in need of further imaging biomarkers, to aid in diagnosis and subsequent monitoring.

Posttraumatic Stress Disorder

PTSD is a trauma and stressor-related disorder that results in complex somatic, cognitive, affective and behavioural effects, after exposure to a traumatic event(s). It is precipitated by a number of factors, including exposure to actual or threatened death or serious injury or as a response to intense fear, helplessness or horror. PTSD is characterised by persistent re-experiencing of the traumatic event; avoidance of stimuli related to the trauma; and hyperarousal for at least one month post trauma (American Psychiatric Association 1994), leading to significant psychosocial impairment for patients. PTSD is common in the general and military populations with an estimated 12-month prevalence of 5.2% vs. 8.3%, respectively (McFarlane, Hodson et al. 2010).

PTSD is a debilitating psychiatric condition. To date there is no objective test for PTSD, which is diagnosed clinically. A MRS biomarker would not only be helpful for diagnosis in PTSD but may be helpful for identifying individuals at risk of developing PTSD, monitoring therapeutic response and

determining disease severity. As part of this study a systematic review of previous literature is performed utilising MRS to characterise differences in neurometabolites in the brain of PTSD patients.

MS is a debilitating neurological condition which is thought to be due to autoimmune mediated demyelination. Unlike PTSD, MRI has become a critical component in the diagnosis and monitoring of MS. However, there are limitations to what information conventional MRI (T1 and T2 weighted images) can provide. There is now a need to identify a technique that allows clinicians to non-invasively understand the pathologic changes associated with disease progression and to evaluate the efficacy of treatments on the pathologic cascade of MS.

Relapsing and Remitting Multiple Sclerosis

MS is a chronic immune-mediated demyelinating condition. Disease severity is monitored using clinical symptoms and MRI of the brain. In recently published guidelines for the management of MS cases, brain and spinal cord MRI have a prominent role in the diagnosis and follow up of dissemination in time and space of central nervous system involvement (Traboulsee, Simon et al. 2016). In day-to-day practice radiologists monitor patients' brains for new T2 intense lesions, with or without evidence of diffusion restriction and/ Gadolinium contrast enhancement, as a marker of active demyelination. There is increased reliance on MRI in MS disease management, however, conventional techniques are limited in their accuracy as a primary outcome measure of efficacy in new therapeutic trials and for disease severity in general. There is a clinico-radiological mismatch, such that there can be a poor association between clinical findings and radiological disease severity (Barkhof 2002, Chard and Trip 2017, Healy, Buckle et al. 2017). Next to multifocal white matter lesions, other contributing pathological features remain unrecognised due to the limitation of conventional MRI protocols (such as T1 and T2 weighted imaging) to detect CNS changes including functional alterations in normal appearing brain matter (NABM). Changes in normal appearing brain parenchyma, gray matter lesions (Geurts, Reuling et al. 2006) and neurodegeneration lead eventually to brain atrophy and accumulating disability during the course of the disease (Bermel and Bakshi 2006, Roosendaal, Bendfeldt et al. 2011). In addition, following the explosion in the number of new disease modifying therapies available for the treatment of MS in the last decade, it has become essential to better understand the underlying pathological changes associated with disease progression and thereby develop new meaningful imaging biomarkers to radiologically evaluate the clinical efficacy of treatments.

Hypotheses and Aims

Aim 1: To assign fucose-α(1–2)-galactose residues and the substrate α-L-fucose in the human brain using 2D L-COSY technology.

The introduction of *in vivo* 2D spectroscopy, in conjunction with technological advances in water suppression, has allowed the current study to make unambiguous assignments, such as that of fucosylated glycans (see Chapter 2) that could not have been made using 1D spectroscopy alone and has also increased the clinical utility of *in vivo* MRS. Fucose was initially identified to be important in malignancy, where it is known to play a key role in haematogenous metastasis (Lean, Mackinnon et al. 1992). In this thesis, the first *in vivo* evidence of **α**-L-fucose (from here in referred to as Fucose) in the human brain is provided. In the brain, Fucose is thought to be primarily contained within the synapsin proteins (Hart 2006), which are thought to regulate the release of neurotransmitters at the synapse (Evergren, Benfenati et al. 2007). Fucosylation prevents the rapid degradation of these proteins (Hart 2006). Interestingly, Revest, Kaouane et al. (2010) found that blocking the fucosylation of synapsin Ia/Ib inhibits the glucocorticoid mediated increase in stress-related memories (Revest, Kaouane et al. 2010) in the hippocampus, raising the possibility that this molecule may have a role in PTSD. Additionally, neuronal glycan proteins modified with fucose, have been shown to be important in learning and memory (Hart 2006) and are implicated in synaptic plasticity.

Aim 2: Develop and evaluate methods for partial volume correction of 1D and 2D MRS and to develop a method for the extraction of metrics of interest (such as diffusion) from a MRS voxel.

Post processing is a critical component of the analysis of both 1D and 2D MRS. Metabolites can be measured using either relative or absolute quantification. Absolute quantification is recommended by many authors as the most accurate method, and does not become unreliable with changing brain parenchymal creatine. Absolute quantification of metabolites is reliant upon tissue water concentration as an internal reference, however, this varies according to the tissue contained within the MRS voxel. If the water concentration is not corrected prior to metabolite quantification, metabolite concentrations may be underestimated (Lee, Caparelli et al. 2013). Chapter 3 gives a detailed, step-by-step guide on how to perform partial volume correction for 1D and 2D spectroscopy and how to quantify other parameters of interest from within a MRS voxel, such as fluid attenuated inversion recovery (FLAIR) hyperintensities, in cases of demyelination, or diffusion weighted imaging metrics. As part of this contribution, code is developed to automate the process of partial volume

correction and extraction or imaging data from a MRS voxel. The techniques that are described in Chapter 3 are then implemented in Chapters 4 and 5.

Aim 3: Systematically review studies that have characterised neurometabolite alterations in PTSD using MRS.

In Chapter 4 MRS studies investigating PTSD are systematically reviewed. This systematic review summarises the results of 24 MRS studies, performed between 1998 and 2017, to measure neurochemical changes occurring in the human brain as a consequence of PTSD. The most consistent finding in subjects with PTSD is reduced N-acetylaspartate (NAA) levels in the hippocampus and anterior cingulate cortex (ACC), which was found with and without atrophic change. More recent studies, using advanced spectroscopy techniques and modern hardware, have shown evidence of glutamatergic dysfunction and changes in gamma-aminobutyric acid levels in the brain of patients with PTSD. Conflicting results have been reported in choline-containing compounds and there is emerging evidence of myo-inositol and glutathione being disturbed in PTSD. The review highlights the need for further studies using new technologies, such as 2D L-COSY to further characterise PTSD.

Aim 4: To characterise neurochemical alteration in the brain, in patients with PTSD using 2D L-COSY.

In Chapter 4, the current study utilising L-COSY to investigate PTSD is detailed, comparing it to conventional 1D spectroscopy. The hypothesis was that 2D spectroscopy would provide additional metabolic information, not available using traditional 1D MRS. It was then determined if metabolic dysregulation in PTSD was associated with clinical symptoms. Also an aim was to determine if there were unique metabolite alterations in the posterior cingulate cortex, a highly connected component of the limbic network, in participants with PTSD when compared to healthy controls. Then it was determined if any of the metabolites identified were associated with clinical symptoms including the clinical assisted PTSD severity score. The 2D MRS method, 2D L-COSY, identified specific neuro deregulation not previously recorded in PTSD. This included an increase in two fucose-α(1–2)-glycans as well as the appearance of the substrate α-fucose. The fucose-α(1–2)-glycans have been implicated in the molecular mechanisms that underlie neuronal development, learning and memory using animal models (Murrey, Ficarro et al. 2009). This is the first evidence of fucose-α(1–2)-glycan involvement in the pathogenesis of PTSD in the human brain. Other metabolic differences recorded in this study include: an increase in imidazole from either histamine, histidine or homocarnosine (IMI-1) and an increase in the level of unsaturation in a lipid fatty acyl chain. Differences in IMI-1 were correlated with clinical symptoms of PTSD. Using 1D MRS, reduced inositol in the posterior cingulate cortex (PCC) was

identified, which was positively correlated with hyperarousal symptoms. Additionally, 1D MRS identified increased glutamate in the ACC, raising the possibility that increased glutamate may result in excitotoxicity in this region.

Aim 5: To characterise neurochemical alteration in the brain, in patients with relapsing and remitting multiple sclerosis, using 2D L-COSY.

In Chapter 5, 2D L-COSY is applied to the brains of patients with relapsing and remitting MS for the first time. The hypothesis was that 2D L-COSY could identify novel biomarkers to monitor disease activity in normal appearing brain parenchyma, not available using other techniques. The aim was to identify neurochemical differences in the normal appearing brain matter of clinically stable relapsing remitting MS patients, compared to age and gender-matched healthy individuals, using 2D L-COSY. It was determined if any of the metabolites identified were associated with clinical symptoms including disability status, cognitive function, mood status and fatigue. Associations between features derived from more conventional MRI approaches were compared, including brain volume estimates and lesion load with clinical outcomes, thereby evaluating the benefits of obtaining additional information regarding alterations in metabolic species in the MS brain by L-COSY.

Despite the mean whole brain lesion volume being low in this RRMS group (6.8mls), a significant reduction in PCC metabolite to tCr ratios were identified for multiple N-acetylaspartate (NAA) signatures, gamma-aminobutyric acid (GABA), glutamine and glutamate (Glx), threonine and isoleucine/lipid. Of the clinical symptoms measured, visuospatial function, attention and memory were correlated with NAA signatures, Glx and isoleucine/lipid in the brain. NAA is closely correlated with most atrophy measures ($r=0.43$; $p<0.003$). Grey matter volume (GMV) and total lesion volume (TLV) are most closely associated with cognitive function (GMV: 0.39; TLV: -0.401; $p<=0.02$) and disease severity (GMV: -0.49; TLV: 0.46; $p<=0.001$).

Chapter 2

Six Fucose-α(1−2) Sugars and α Fucose Assigned in the Human Brain using In Vivo Two Dimensional Magnetic Resonance Spectroscopy at 3T

Scott Quadrelli, Carolyn Mountford, Alexander Lin, Saadallah Ramadan

NMR Biomed. 2015 Mar;28(3):291-6. doi: 10.1002/nbm.3239. Epub 2014 Dec 22.

Abstract

A growing body of literature has indicated that fucose-α(1–2)-galactose sugars are implicated in the molecular mechanisms that underlie neuronal development, learning and memory in the human brain. An understanding of the in vivo roles played by these terminal fucose residues has been hampered by the lack of technology to non- invasively monitor their levels in the human brain. We have implemented in vivo two-dimensional MRS technology to examine the human brain in a 3-T clinical MR scanner, and report that six fucose-α(1–2)-galactose residues and free α-fucose are available for inspection. Fucose-α(1–3)-galactose residues cannot yet be assigned using this technology as they resonate under the water resonance. This new application offers an unprecedented insight into the molecular mechanisms by which fucosylated sugars contribute to neuronal processes and how they alter during development, ageing and disease.

Introduction

L-Fucose is a hexose deoxy sugar with the chemical formula C6H12O5. It is found on N-linked glycans on the mammalian cell surface. Two structural features distinguish fucose from other six-carbon sugars present in mammals: the lack of a hydroxyl group on the carbon at the 6-position (C-6), and the L-configuration. In the fucose-containing glycan structures, fucose can exist as a terminal modification or serve as an attachment point for adding other sugars. The enzyme α-L-fucosidase removes L-fucose residues from the non-reducing terminus of the glycan chain and fucosyltransferase catalyses the transfer of L-fucose from the guanosine diphosphate β-L-fucose (GDP-fucose)(Becker and Lowe 2003, Tu, Lin et al. 2013).

α-L-Fucose is usually expressed as a terminal saccharide on N- and O-linked glycoproteins and glycolipids (Becker and Lowe 2003). In this article we will refer to α-L-fucose as “fucose”. Being terminal, fucose is usually mobile on the Magnetic Resonance (MR) timescale. In a one-dimensional (1D) MR spectrum the resonances from different molecular components often overlap making assignment difficult. Two-dimensional (2D) MR methods overcome this by adding a second frequency dimension, acquiring multiple 1D spectra with incrementally longer echo times (TE) and applying a double Fourier transform on the set of spectra to produce a 2D spectrum. The pulse sequence implemented is based on a series of slice selective radio frequency (RF) pulses (90-180-t1-90-acquire, 90: sinc shaped, 180: Mao shaped) as initially reported by Thomas et al (Thomas, Yue et al. 2001). In this sequence, the first two RF pulses are responsible for generating a spin echo signal, while the final 90 RF pulse is responsible for slice selection and coherence transfer. The resonances on the diagonal of the 2D spectrum provide

the same information as the 1D spectrum. Those resonances on the diagonal that are connected by an off diagonal cross peak are scalar coupled and allow these molecules to be assigned unambiguously. This technology is commonly used by chemists to study the structure of isolated molecules and proteins in solution.

Lean et al first used 2D MR spectroscopy to assign fucosylated glycans in human malignant colorectal cancer cells (Lean, Mackinnon et al. 1991). Human malignant colon cancer cell models allowed the fucose assignments to be confirmed using 2D Correlated SpectroscopY (COSY) in two ways. Firstly, treatment with the enzyme fucosidase and acid hydrolysis of the cells (Wright, May et al. 1988) allowed the assignment of five terminal fucose moieties as well as free α and β fucose (Lean, Mackinnon et al. 1991, Lean, Mackinnon et al. 1992). Secondly, increases in the cross peak volumes were found following the addition of free fucose to the cells (Lean, Mackinnon et al. 1992). In biologically well-defined cell models, the fucose patterns were clearly correlated with the extent of cellular differentiation (Mountford, Doran et al. 2004), with a gradual appearance of molecules denoted Fucose (Fuc) I to Fuc IV. The 2D COSY method was subsequently applied to examine the extent of de-differentiation in human biopsy specimens in a blinded study and the result confirmed by histopathology (Mountford et al unpublished data). It was considered but not proven at the time that the origin of these fucose molecules was the Lewis Y and Lewis X antigens known to be expressed in human malignant cells.

Others have since used 2D MR spectroscopy to study isolates of oligosaccharides to determine the structure of the blood group antigens that contain terminal fucose residues (Meloncelli, West et al. 2011). They reported that the MR spectral characteristics, in particular chemical shift, alter according to the type of oligosaccharide the fucose has been incorporated into (Meloncelli, West et al. 2011). They also describe how the chemical shifts of these fucose entities can alter with changes to microenvironment (Klein, Lamblin et al. 1988, Van Halbeek 1988, Meloncelli, West et al. 2011), such as pH. The spectral characteristics are consistent with several of those assigned in the cell models.

There is now evidence that Fucose-α(1−2)-galactose sugars are implicated in the molecular mechanisms that underlie neuronal development, learning, and memory (Murrey, Ficarro et al. 2009). Early evidence also suggests a pivotal role for these glycans in regulating nervous system development and function (Murrey and Hsieh-Wilson 2008). Others have demonstrated that glycosylation influences various neuronal processes, such as neurite outgrowth and morphology (Kleene and Schachner 2004, Murrey, Ficarro et al. 2009).

Until now, an understanding of the precise role(s) of fucose in the human brain has been hampered by our inability to monitor these glycobiological processes in vivo. However in vivo MR technology has developed sufficiently to allow the 2D MRS method to be applied to examine the human brain. There are several protocols available, however the one employed here is Localised Correlated SpectroscopY (L-COSY) which provides an insight into biochemical mechanisms not possible by any other in vivo technique thus far (Ramadan S, Andronesi O et al. 2011). Importantly, in vivo L-COSY can identify which specific chemicals are altering in the brain and their relationships to each other.

Our group has now used the L-COSY method for many human brain *in-vivo examinations*. We have noted that it is usual for this spectral region (F2: 3.95 - 4.50 ppm and F1: 0.90 - 1.70 ppm) to be populated, albeit differently between some subjects. Here we assign six cross peaks consistent with Fucose-α(1−2)-galactose residues and the substrate α-L-fucose.

Magnetic Resonance Imaging and Spectroscopy Protocols

Data were acquired on a Siemens 60cm bore Trio (Siemens AG, Erlangen, Germany) using an 8 channel head coil. The head was secured in the coil with foam pads to reduce patient movement.

Structural imaging

T1-weighted MPRAGE volumetric sequence (TR/TE=2530/1.7 ms, 12 degree flip angle, Field Of View = 256x256x256 mm, slice thickness 1mm, voxel size 1x1x1mm, Number of Experiments = 4, acquisition time 6 minutes). Prior to the L-COSY spectroscopy data collection, routine brain MRI was performed with axial 3D-MPRAGE and reconstructed in the sagittal and coronal planes with 2 mm slice resolution for accurate localization of the voxel. The posterior cingulate cortex (PCC), predominantly comprising grey matter was chosen as this gave the better spectra. No segmentation was undertaken.

2D L-COSY

L- COSY was acquired in the PCC (size 3x3x3 cm3), with the following parameters: RF carrier frequency at 2.0 ppm; TR 1.8 s; water suppression using WET; spectral width of 2000 Hz; increments size of 0.8 ms in 96 t1 increments giving an indirect spectral width of 1250 Hz; 12 averages per increment; and 1024 data points. Scan time for the 2D L-COSY was 35 minutes.

Subjects

Eighteen volunteers were recruited for this study with an age range of 22 to 55. The study was approved by the local institutional review boards and was compliant with the Health Insurance Portability and Accountability Act. All subjects provided informed consent.

Data Processing

Raw L-COSY data were transferred to MATLAB (MathWorks 1984-2014) for signal combination from multiple elements followed by row concatenation into a 2D matrix. Commercial 2D spectral processing software (Felix-2007, Accelrys, San Diego, CA, USA) was used for observer-independent spectral processing and analysis. The processing parameters used were: F2 domain (skewed sine-squared window, 2048 points, magnitude mode), F1 domain (sine-squared window, zero-filling to 512 points, magnitude mode). The effect of altering time domains and window functions in 2D COSY has been documented elsewhere (Delikatny, Hull et al. 1991). Residual water was removed by using a Felix built-in Gaussian shaped convolution-based method. The total creatine methyl diagonal resonance at 3.02 ppm was used as an internal chemical shift reference in F1 and F2. All 'cross' or off-diagonal peaks are denoted with F2 – F1 in ppm units. The volumes of cross peaks or diagonal resonances were evaluated using Felix software described above, and care was taken to ensure that the volume evaluated was the same in all 2D spectra. All identifiable peaks according to Lean (Lean, Mackinnon et al. 1991, Lean, Mackinnon et al. 1992) or new assignments were reported, and peaks were measured and normalized to the creatine diagonal peak volume at 3.02 ppm for comparable results across all data sets. Cross peaks were deemed to be present if the peak volume was 4.8 times higher than the volume of an area of noise measured in a spectral region where no cross peaks were apparent.

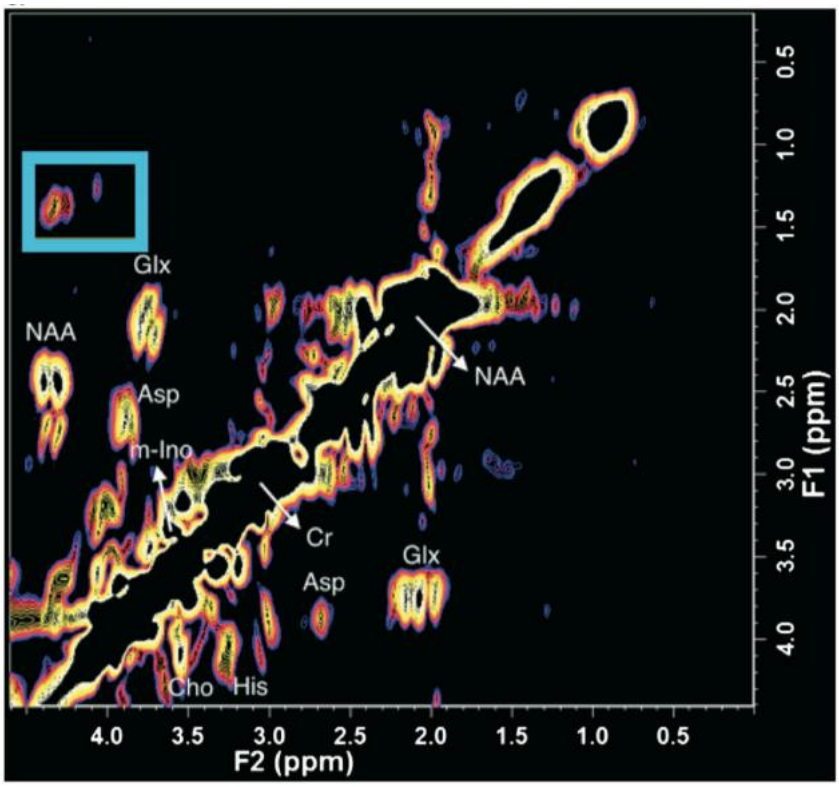
Results

A typical two dimensional contour plot of an L-COSY data set, recorded from the posterior cingulate cortex of a male brain, is shown in Figure 2A with assignments as described in Ramadan et al (Ramadan S, Andronesi O et al. 2011). In Figure 2B a three-dimensional plot of the same spectrum is shown where the intensity and relative volume of each cross peak is more clearly seen. The region containing the fucose molecules (F2: 3.95 - 4.50 ppm and F1: 0.90 - 1.70 ppm) is shown expanded in Figure 3A as a contour plot, and in Figure 3B as a three-dimensional plot.

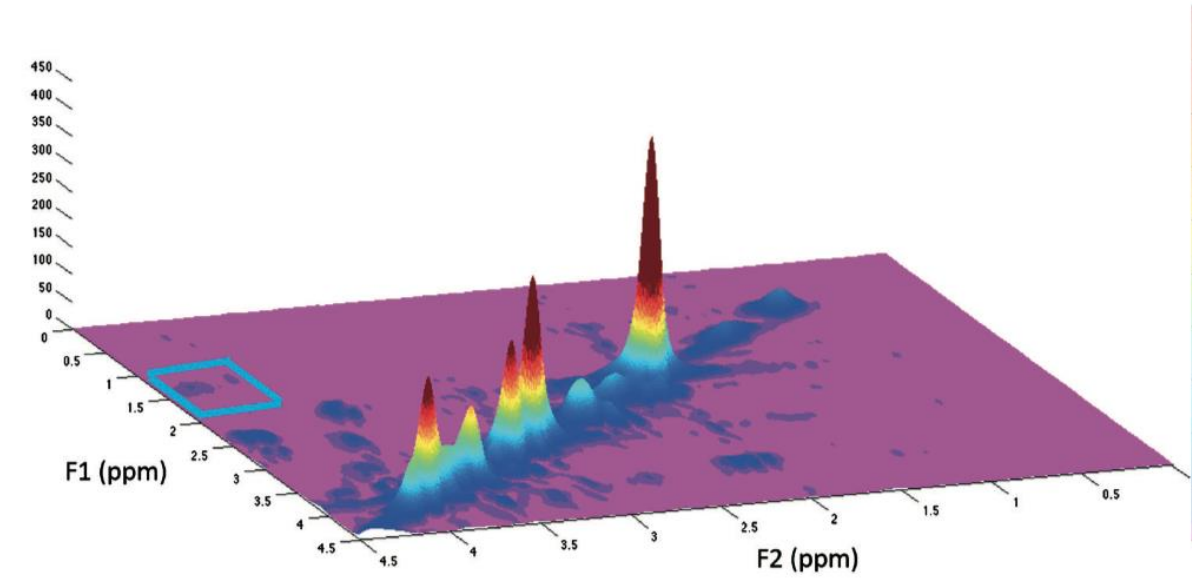
The cross peaks are assigned using the same nomenclature as reported by Lean et al (Lean, Mackinnon et al. 1992), i.e. Fuc I to IV, and summarized in Table 1. Free α-L-Fuc, is also recorded at 4.21 - 1.22ppm. There is a second cross peak at 4.17 - 1.32ppm (Figure 3A) that we tentatively assign to α-Fuc as well. If these are indeed both α-Fuc they could arise from either being in two different environments; or as the same substrate/product but in slow exchange between two different environments, i.e. its movement between two sites is so slow that a signal is generated from each site. Lactate is recorded in this particular brain spectrum, but is not present in all subjects (Ross JM, Öbergc J et al. 2010).

Two other cross peaks, not shown in figure 3A, have been recorded. Fuc II as previously assigned in cell models, at 4.28 - 1.14 ppm and Fuc V at 4.36 - 1.58ppm, not reported before in cell lines or biopsies.

A


Glx
NAA
Asp
m-Ino
NAA
Cr
Asp
Glx
Cho
His
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
F2 (ppm)
F1 (ppm)

B


F1 (ppm)
F2 (ppm)

C

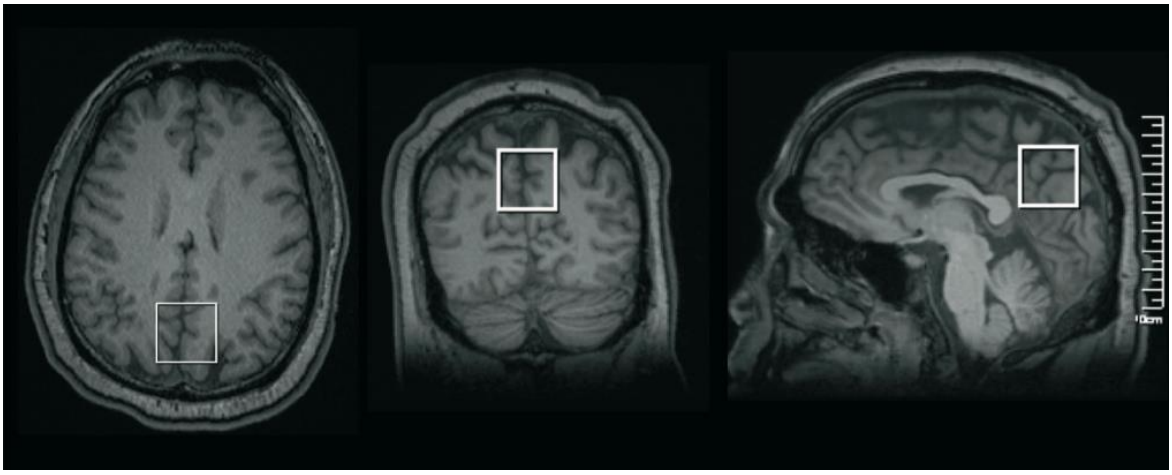
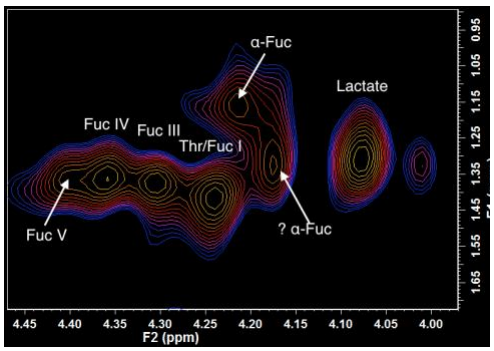


Figure 2 - In Vivo L-COSY of the human brain (PCC) acquired at 3T using a 8 channel head coil; voxel size 30x30x30 mm3, increment size 0.8ms, increments 96, 12 averages per increment, TR 1.8 sec, total experimental time 35 min, acquired vector: 1024 points, acquisition time: 512 ms, spectral width in F2: 2000 Hz, spectral with in F1: 1250 Hz. Abbreviations: N-acetylaspartate (NAA), choline (Cho); creatine (Cr); glutamate and glutamine together (Glx); aspartate (Asp); myoinsitol (m-Ino); histidine (His). The region highlighted by the blue box is expanded in Figure 2. A) Contour plot produced using Felix software. B) Three-dimensional plot of the same data set produced using Matlab. C) Representative voxel location in a healthy volunteer.

A



B

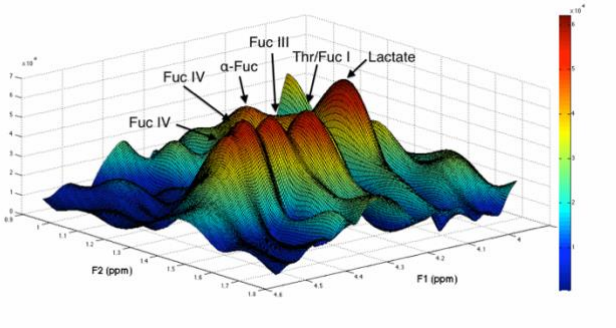


Figure 3 - Expanded region (F2: 3.97 - 4.47, F1: 0.90 - 1.72;) of the L-COSY in Figure 1 with assignments of Fuc I and FucIII to Fuc V and α-L-Fucose denoted. This patient did not have a Fuc II peak present. The cross peak labeled ? α-L-Fucose at 4.17 - 1.33ppm we are proposing may be an α-L-fucose either in a different environment or in slow exchange with cross peaks labeled α-L-Fucose assigned at 4.21 - 1.16ppm. A) Contour plot produced using Felix software. B) Three-dimensional plot of the same data set produced using Matla

Table 1 - Assignments and Chemical Shifts of Molecules in the Spectral Region F2: 3.90-4.60, F1: 0.90-1.75.

Assignment	*In-vitro* (Lean, Mackinnon et al. 1992) (F2, F1)	*In-vivo* (F2, F1)	95% Confidence Interval	Comparison with published isolates
Thr/Fucose-1	4.27 - 1.33	4.25 - 1.38	± (0.02 - 0.02)	
Fucose II	4.28 - 1.25	4.28 - 1.14	± (0.03 - 0.06)	Fuc α1-2 (Strecker G, Wieruszeski JM et al. 1992) Fuc α1-2 (Dua, Rao et al. 1986, Klein, Somorjai et al. 1988, Van Halbeek 1988)
Fucose III	4.30 - 1.41	4.31 - 1.37	± (0.01 - 0.01)	
Fucose IV	4.38 - 1.31	4.36 - 1.36	± (0.01 - 0.04)	Fuc α1-2 (Strecker G, Wieruszeski JM et al. 1992)
Fucose V		4.40 - 1.36	± (0.07 - 0.05)	
α-L-Fucose	4.21 - 1.22	4.21 - 1.16	± (0.02 - 0.04)	
? α-L-Fucose		4.17 - 1.33		
Lactate	4.10 - 1.30	4.08 - 1.29	± (0.01 - 0.03)	

Discussion

The improvement in MR technology is now such that a non-invasive window into neuro-glycochemistry is available using a clinical 3T scanner and the L-COSY MR spectroscopy method. We have identified and assigned six fucose residues in spectra from the PCC region in the human brain. There is variation in this spectral region from person to person according to their health state and or age (Maudsley, Domenig et al. 2009). Unlike the cell models, where these initial assignments were verified by controlled enzymatic cleavage, this is clearly not possible in vivo. There are however no other obvious potential contenders for assignment in this region except for the amino acid threonine which is at the same frequency as Fuc I, and which must therefore be considered a composite cross peak. Importantly the chemical shifts we report here for fucose residues are consistent with the reports by others using the 2D MRS method to examine oligosaccharides extracted from blood antigens mucosa and natural products (Strecker G, Wieruszeski JM et al. 1992).

Assignment of Fucα 1-2 glycans as Reported in Isolated Lewis Antigens

Strecher et al (Strecker G, Wieruszeski JM et al. 1992) isolated three acidic oligosaccharide-alditols carrying Lewis X, Lewis Y and A-Lewis Y determinants from the jelly coat of *Pleurodeles waltl* eggs. The three compounds in their isolates were Gal beta 1-4(Fuc alpha 1-3)GlcNAc beta 1-3[2-oxo-3-deoxy-D-glycero-D-alactononulosonic acid (KDN)alpha 2-6] GalNAc-ol; Fuc alpha 1-2Gal beta 1-4(Fuc alpha 1-3)GlcNAc beta 1-3(KDN alpha 2-6) GalNAc-ol; and Fuc alpha 1-2(GalNAc alpha 1-3)Gal beta 1-4(Fuc alpha 1-3)GlcNAc beta 1-3(KDN alpha 2-6)GalNAc-ol. They also used 2D MR spectroscopy to assign two αFuc1-2 molecules at 4.26-1.27ppm and 4.34-1.30ppm. These are consistent with Fuc II and Fuc IV assigned in the cell models and in the human brain (Table 1). Three Fucose-α(1-3) molecules were also assigned in that study but they resonate under the water resonance in the human brain L-COSY at 4.8ppm. Assignment of Fucose-α(1-3) in the human brain requires further protocol development.

There is now a large and detailed body of literature on the structures and conformational differences exhibited by the blood group antigens with terminal fucose structures. Meloncelli et al studied 18 ABO type I–VI antigens containing fucose (Meloncelli, West et al. 2011) following controlled synthesis of the type III and IV antigens. They undertook MR spectroscopy studies to probe differences in overall conformation of the molecules. Their findings are important to explain the range of chemical shifts of the Fucose-α(1−2) glycans reported here in the human brain. While their results showed little difference in the MR chemical shifts suggesting nearly identical conformations in solution, there was one major exception and that was the terminal fucose residue where deviations of 0.33 ppm from the

4.32ppm average were reported. Klein (Klein, Lamblin et al. 1988) and Van Halbeek (Van Halbeek, Breg et al. 1988) showed that the chemical shift of fucose was sensitive to the linkage type of local residues in the same molecule. They also suggested that the shift effects in the MR spectrum from different determinants provides a reliable means for identifying the linkages, and that terminal fucose residues are sensitive to changes in structure as the molecules form in vitro (Dua, Rao et al. 1986, Klein, Lamblin et al. 1988).

Thus by comparing these in vivo 2D neuro-spectroscopy results with the results from the blood group antigens, it supports the assignment of Fucose-α(1-2) residues. The range of chemical shifts recorded for the Fucose-α(1-2) residues, assigned to Fuc I, Fuc III, Fuc IV and Fuc V, in the human brain can thus be attributable to a different composition of the oligosaccharide chains in which they reside, or a different conformation (Klein, Lamblin et al. 1988).

Free alpha–L-Fucose

Fucosylated glycans are constructed by fucosyltransferases, which require the substrate GDP-fucose. Two pathways for the synthesis of Guanidine 5′-diphosphate-β-l-fucose (GDP-fucose) operate in mammalian cells, the GDP-mannose-dependent de novo pathway and the free fucose-dependent salvage pathway (Becker and Lowe 2003). There is a cross peak at 4.21 - 1.16ppm in some of the human brain spectra which is at the expected chemical shifts for free α fucose and is thus assigned as such. However, there is a cross peak at 4.70 - 1.30, not yet assigned, which may be a second free α fucose in either a different environment or possibly in slow exchange with that assigned at 4.17 - 1.33ppm i.e. its movement between two sites is so slow that a signal is generated from each site.

No cross peak at the chemical shift for β-fucose was observed indicating that the β-fucose substrate is in intermediate exchange in the MR timeframe and thus the resonance too broad to observe. The observation of free fucose in the spectra indicates that at least one of these six fucose moieties relies on the salvage pathway. These are interesting observations which require more investigation, but if correct may offer the opportunity to monitor the dynamics of fucosylation *in* vivo in response to disease states or to therapy.

Fucose (α-Fucose) also plays a central and well-known role in the inflammatory cascade. Fucose functions as a carbohydrate ligand for the Selectin family of cell adhesion/signalling receptors. Selectins are located on the surface of endothelial cells, platelets and leukocytes where they regulate aspects of the cascade such as adhesion, rolling and transmigration of leukocytes (Becker and Lowe 2003, Listinsky, Siegal et al. 2011). The disaccharide motif Fucα(1-2)Gal is also thought to be involved in

processes such as asthma, and is known to be part of the tumorigenesis process (Becker and Lowe 2003). We have early evidence now that several of these Fucα(1-2) molecules alter with chronic pain. It is thus conceivable that that Fucα1-2 may be relevant to the inflammatory process, and is now available for inspection.

Fucose-α(1-2)-galactose in the Brain

To better understand the role of Fucose-α(1-2)-galactose, animal models have been studied. Fucose-α(1-2)-galactose has been implicated in the cognitive processes of learning and memory, and electrophysiological models of learning and memory induced protein fucosylation in hippocampal neurons (Pohle W, Acosta L et al. 1987). The introduction of free α fucose enhanced long term potentiation in vivo (Krug M, Wagner M et al. 1994, Matthies, Staak et al. 1996). This was reversed using inhibition by 2-deoxy-D-galactose (2-dGal) which caused reversible amnesia (Lorenzini CGA, Baldi E et al. 1997). Fucose-α(1-2)-galactose is shown to affect, and could even regulate, neurite outgrowth and neuronal morphology. Delayed synapse formation and stunted neurite outgrowth were observed in hippocampal cultures inhibited with 2-dGal (Kalovidouris SA, Gama CI et al. 2005, Murrey HE, Gama CI et al. 2006) whereas the Fucose-α(1-2)-galactose sugars stimulated neurons, and other sugars such as Fuc(1-3)-N-acetylglucosamine had no effect (Kalovidouris SA, Gama CI et al. 2005).

Murrey et al identified a "Plasticity-Relevant" Fucose-α(1−2) galactose proteome from the mouse olfactory bulb (Murrey, Ficarro et al. 2009). They showed five classes of putative Fucose-α(1−2)-galactose glycoproteins: cell adhesion molecules, ion channels, solute carriers/transporters, ATP-binding proteins, synaptic vesicle-associated proteins, and mitochondrial proteins. They also reported that Fucα(1−2)Gal glycoproteins were enriched in the developing mouse olfactory bulb and exhibited a specific spatiotemporal expression consistent with the presence of a "glycocode" to help direct olfactory sensory neuron axonal path finding.

This L-COSY technology is directly applicable to animals and by combining approaches it will accelerate the discovery of new Fucα(1-2)Gal glycoproteins and provide new insights into the molecular mechanisms by which fucosyl sugars contribute to neuronal processes in neurobiology. The study of neuronal development in animal models (Chaubard, Krishnamurthy et al. 2012) would permit direct comparison with the new chemo-enzymatic strategy that detects Fucose-α(1-2)galactose. The strategy the Caltech group (Chaubard, Krishnamurthy et al. 2012) has developed is a means of detecting a variety of complex Fucose-α(1-2)galactose glycans and glycoproteins allowing living cells and complex tissue extracts to be studied. Studies to determine how these Fucose-α(1-2)galactose molecules are

perturbed by chronic pain, head injury and Posttraumatic Stress Disorder are currently underway, as are studies of the glycobiology of Fucα(1-2)Gal during adolescence.

Technical Considerations

The data was recorded in a clinical 3T MR scanner with a 60cm bore magnet and took 35 minutes to accrue using an 8 channel head coil(Ramadan S, Andronesi O et al. 2011). It is important during the setup of the acquisition parameters to ensure adequate water suppression, as the Fucose-α(1-2)galactose resonances are close to the water signal. Software version VD13A on the Siemens operating system was amended to facilitate greater control of the water signal.

This current protocol incorporates a relatively large voxel and takes a substantial time to collect the 96 experiments required to ensure adequate signal to noise and resolution in both F2 and F1. This is necessary to measure the chemical shifts with reliability in the fucose region. We did not undertake segmentation of grey and white matter, the voxel interrogated includes both. The spectral quality of other regions in the brain was less than optimal.

A higher Bo might prove helpful for improving spectral dispersion, however it comes along with the additional burden of shorter T2 and longer T1. Bo and B1 inhomogeneities also increase and need to be addressed especially at 7T fields and above. It is noteworthy that in cell models a frequency dependence was recorded for these fucose resonances at 11.8T (Lean and Mountford, unpublished data)

Conclusions

In vivo L-COSY MR spectroscopy of the human brain can identify several Fucose-α(1-2)-galactose species, present in relatively small amounts compared to the major neurotransmitters NAA and glutamate. There are several Fucose-α(1-2)-galactose residues at slightly different chemical shifts, indicative of partial structures as the molecules are formed in vivo and/or different linkages to lipids or proteins. This method offers the opportunity to monitor molecular mechanisms by which fucosylated sugars contribute to neuronal processes and how they alter during development, ageing and disease.

Acknowledgements

We thank Valerie Graves for management of the ethics compliance in both countries, Drs Cynthia Lean, Wanda Mackinnon and Lesley Wright for their early and dedicated work assigning these molecules in vitro, and Jessica Buck for preparation of the manuscript. This research was conducted with the early support of a grant from the Australian Research Council (DP0663987). The technology benefited from

Discovery Grant (DP0663987) from the Australian Research Council; US DoD CDMRP (W81XWH-10-1-0835); and Draper Laboratory.

Chapter 3

Hitchhikers Guide to Voxel Segmentation for Partial Volume Correction of *in-vivo* Magnetic Resonance Spectroscopy

Scott Quadrelli, Carolyn Mountford, Saadallah Ramadan

Magnetic Resonance Insights. 2016; 9: 1–8. Published online 2016 Apr 27. doi: 10.4137/MRI.S32903

Abstract

Partial volume effects have the potential to cause inaccuracies when quantifying metabolites using proton magnetic resonance spectroscopy (^{1}HMRS). In order to correct for cerebrospinal fluid content, a spectroscopic voxel needs to be segmented according to different tissue contents. This chapter aims to detail how automated partial volume segmentation can be undertaken and provide a software framework for researchers to develop their own tools. Whilst many studies have detailed the impact of partial volume effects on proton magnetic resonance spectroscopy quantification, there is a paucity of literature that explains how voxel segmentation can be achieved using freely available neuroimaging packages. Additionally, we consider how to correct for partial volume effects in 2D L-COSY. Finally, we look at how other neuroimaging metrics can be extracted from a spectroscopy voxel, such as diffusion weighted imaging indices or white matter lesions for example.

Introduction

Proton magnetic resonance spectroscopy (MRS) recorded from the brain allows for non-invasive quantification of tissue metabolites that are visible on the MR time-scale, using a conventional MRI scanner. In the brain MRS has been applied to multiple different disease processes such malignancy, multiple sclerosis, cognitive impairment and stroke (Oz, Alger et al. 2014). MRS provides information on tissue metabolism, in both health and disease, and has the potential to provide biomarkers for pathologies not traditionally appreciated on conventional imaging (Foerster, Petrou et al. 2012, Murray, Przybelski et al. 2014, Ramadan, Arm et al. 2015) .

To obtain MRS data, a three-dimensional volume known as a 'voxel' is placed in an area of interest in the brain. A voxel is usually made up of three primary quantities: white matter (WM); grey matter (GM) and cerebrospinal fluid (CSF). The spectroscopy voxel shouldn't be confused with the imaging voxel which represents a much smaller volume, typically $1mm^3$ compared to $1cm^3$ for single voxel spectroscopy (SVS). The area under the metabolite spectral resonances that are obtained from within a spectroscopy voxel, are directly proportional to the *in-vivo* concentration of metabolites (Jansen, Backes et al. 2006) that are visible on the MR time scale. However, the metabolite signal intensity is effected by many factors such as RF coil loading and excitation homogeneity. To correct for these RF effects, metabolite peak intensities need to be normalised against an internal or external standard, commonly leading to expressing the metabolite concentration as a ratio to an internal reference such as total creatine (tCr) or total N-acetylaspartate (tNAA). However, if a change is noted in the

concentration ratio it can be difficult to ascertain if the change is due to the denominator, numerator, or both.

An alternative to relative quantification is absolute metabolite quantification. The most common methods (water signal referencing) for absolute quantification, is reliant upon tissue water concentration as an internal reference (Gussew, Erdtel et al. 2012). The concentration of water varies according to the tissue compartments contained within the spectroscopy voxel. Variations in tissue and CSF contents within the voxel effect quantification in two main ways: the relative water content varies according to the tissue type (ie CSF has a greater concentration of pure water compared to grey matter)(Ernst, Kreis et al. 1993) and the majority of metabolites are contained within the GM and WM (partial volume effects). Therefore, partial volume effects should be considered for accurate quantification. If the water concentration isn't corrected prior to metabolite quantification, metabolite concentrations may be underestimated (Lee, Caparelli et al. 2013). Despite partial volume correction being a recommended step for absolute quantification of metabolites (Jansen, Backes et al. 2006), there is a paucity of literature describing precisely how voxel segmentation can be achieved from a practical standpoint. This guide will be limited to spectroscopy data obtained from a Siemens MRI scanner.

Multi-parametric MRI can be useful in increasing the sensitivity and specificity of a biomarker or to confirm MRS findings. Therefore, it is useful to understand how non-spectroscopy parameters (diffusion weighted imaging (DWI), functional MRI (fMRI) and structural MRI) can be determined within the MRS voxel. The basic steps required to determine alternate MR metrics from within a MRS voxel will be outlined.

Co-registration of voxel mask

A binary mask must first be created that represents the 3D SVS voxel. A binary mask is an image where each voxel contains either 1 or 0, in this case the SVS voxel would be represented by a value of 1 and all other voxels 0. There are multiple ways to create a binary mask, however the 'mask()' function within Matlab(MathWorks 1984-2014) and the Freesurfer (Fischl 2012) application 'mri_volsynth' were used successfully during this work and were chosen for relative ease of implementation.

Prior to performing voxel segmentation, a binary mask representing the spectroscopy voxel must be co-registered to the imaging co-ordinates (i,j,k). The pixels within the voxel mask are transformed using an affine transformation (A), to generate scanner co-ordinates (x,y,z) according to Equation 1:

Equation 1

$$(x, y, z) = A(ijk)$$

We can expand Equation 1 as shown below in Equation 2:

Equation 2

$$\begin{bmatrix} x \\ y \\ z \end{bmatrix} = \begin{bmatrix} r_{xx} & r_{xy} & r_{xz} \\ r_{yx} & r_{yy} & r_{yz} \\ r_{zx} & r_{zy} & r_{zz} \end{bmatrix} \cdot \begin{bmatrix} i \\ j \\ k \end{bmatrix} + \begin{bmatrix} t_x \\ t_y \\ t_z \end{bmatrix}$$

The rotation (*r*) and translation matrices (*t*) can be combined to form a translation matrix:

Equation 3

$$M = \begin{matrix} r_{xx} & r_{xy} & r_{xz} & t_x \\ r_{yx} & r_{yy} & r_{yz} & t_y \\ r_{zx} & r_{zy} & r_{zz} & t_z \\ 0 & 0 & 0 & 1 \end{matrix}$$

Equation 3 can be used to transform structural image from scanner coordinates (x,y,z) to voxel coordinates (i,j,k) with the following transformation matrix:

Equation 4

$$\begin{bmatrix} x \\ y \\ z \\ 1 \end{bmatrix} = M_{MRI} \cdot \begin{bmatrix} i \\ j \\ k \\ 1 \end{bmatrix}$$

Additionally, we can transform the scanner co-ordinates to MRS voxel co-ordinates (i′,j′,k′) using the following transformation matrix:

Equation 5

$$\begin{bmatrix} x \\ y \\ z \\ 1 \end{bmatrix} = M_{MRS} \cdot \begin{bmatrix} i' \\ j' \\ k' \\ 1 \end{bmatrix}$$

Finally, Equation 4 and Equation 5 can be combined to create a translation matrix from anatomical space to SVS space according to Equation 6:

Equation 6

$$\begin{bmatrix} i \\ j \\ k \\ 1 \end{bmatrix} = (M_{MRI})^{-1} M_{MRS} \cdot \begin{bmatrix} i' \\ j' \\ k' \\ 1 \end{bmatrix}$$

The transformation matrix is calculated by taking the product of the inverse anatomical image $(M_{MRI})^{-1}$ transformation matrix and the SVS voxel (M_{MRS}) transformation matrix. The $(M_{MRI})^{-1}$ describes the re-alignment of the voxel coordinates to scanner coordinates and (M_{MRS}) describes the transform of the scanner coordinates to the MRS voxel.

The transformation matrix for the imaging data (M_{MRI}) can be obtained using the SPM command '*V = spm_vol (nii_file)*' which will create a structural array containing image volume data (Ashburner, Barnes et al. 2015).(Ashburner, Barnes et al. 2015). The '.mat' element of the '*V = spm_vol (nii_file)*' array contains the 4x4 affine transformation that maps voxel coordinates to scanner coordinates for the anatomical image. The transformation matrix for the SVS voxel (M_{MRS}) is more difficult to construct and the input data is taken from the scanner exported '.rda' file. Table 2 below outlines the variables required for the '.rda' file and their description.

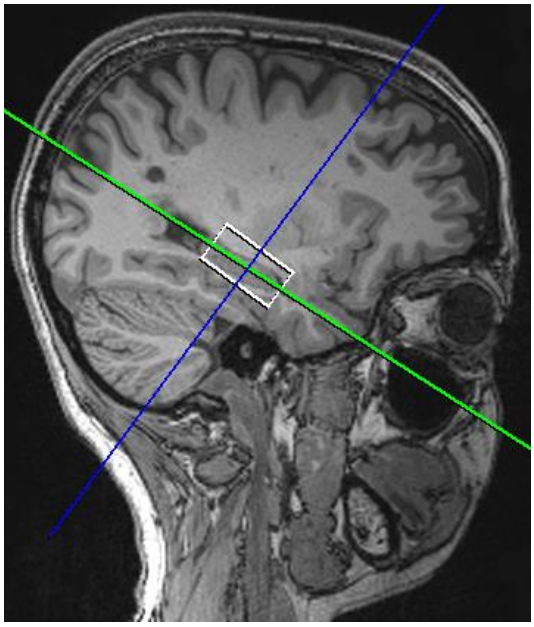
The above steps were performed using a Matlab (MathWorks 1984-2014) function created by (Harris, Puts et al. 2015). The above steps were performed using a Matlab function created by (Harris, Puts et al. 2015), however it is possible to write a custom Matlab (MathWorks 1984-2014) programme using the steps above. It is highly recommended to save screen shots of the voxel location on the MRI scanner for each SVS location, which can be achieved by using 'alt-print screen' key combination and then pasting the imaging into Microsoft Paint on the MRI scanner computer. These reference images can then be used to ensure that the voxel mask created using the steps above, has been correctly transposed into scanner co-ordinates.

Shown below in Figure 4 is an example of a SVS mask overlaid onto a T1-MPRAGE image after the SVS voxel has been transposed to scanner space, for comparison the SVS voxel prescription image at the time of MRI scan is also shown.

Table 2 - A description of the variables contained within the Siemens '.rda' spectroscopy file and how they relate to the voxel dimensions, translation and rotational matrices.

Variable name within .rda file	Description
VOIPhaseFOV, VOIReadoutFOV and VOIThickness	Voxel Dimensions
VOIPositionSag, VOIPositionCor, VOIPositionTra	SVS voxel translation
$\mathbf{ColumnVector[0]}\ \ \mathbf{RowVector[0]}\ \ \mathbf{ColumnVector[0]} \times \mathbf{RowVector[0]}$ $\mathbf{ColumnVector[1]}\ \ \mathbf{RowVector[1]}\ \ \mathbf{ColumnVector[1]} \times \mathbf{RowVector[1]}$ $\mathbf{ColumnVector[2]}\ \ \mathbf{RowVector[2]}\ \ \mathbf{ColumnVector[2]} \times \mathbf{RowVector[2]}$	Rotational matrix

Voxel Prescription Image



Reconstructed SVS voxel

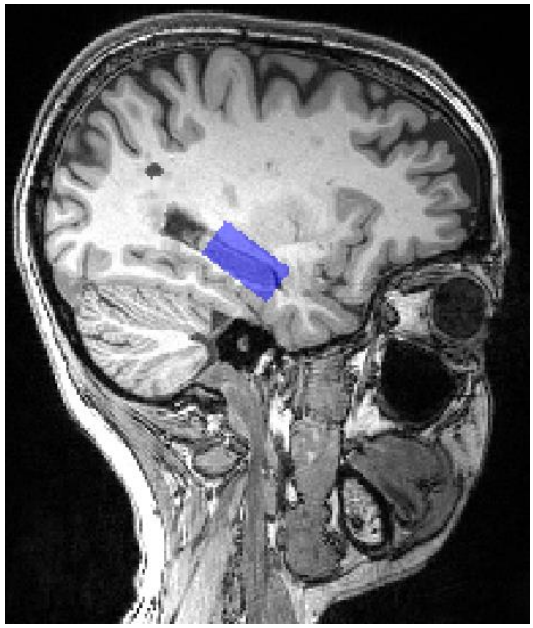


Figure 4 - Top: SVS voxel prescription image taken at the time of scanning. Bottom: Reconstructed SVS voxel displayed as a mask overlying the T1 MPRAGE image. T1 MPRAGE image was acquired at 3T using a 64 channel head coil: (TR/TE/TI=2000/3.5/1100 ms, flip angle = 7°, field of view= 256x256 mm, voxel size 1x1x1mm3).

Segmentation of voxel prescription image

To determine the quantities of tissue subtypes within an SVS voxel, a good quality structural image must be obtained at the time of scanning. A 3D T1 weighted Magnetisation-Prepared RApid Gradient Echo (MP-RAGE) sequence (Brant-Zawadzki, Gillan et al. 1992) allows for identification of anatomical structures at the time of voxel prescription and voxel segmentation at the time of post processing. The following are typical parameters at 3T for a 3D T1-weighted MPRAGE sequence: (TR/TE/TI=2000/3.5/1100 ms, flip angle = 7°, field of view= 256x256 mm, voxel size 1x1x1mm^3, IPAT 2, orientation= sagittal, acquisition time 4:48 minutes).

There are four major open-source neuroimaging packages that allow for segmentation of a 3D brain image into GM, WM and CSF, they are FSL (Smith, Jenkinson et al. 2004), Freesurfer (Fischl 2012), SPM (Ashburner, Barnes et al. 2015) and AFNI (Cox 1996). Freesurfer is limited when it comes to SVS voxel segmentation as sulcal CSF is not segmented (Freesurfer 2015). FSL FAST, which will be referred to as FAST (Zhang, Brady et al. 2001), was chosen for this work, however SPM or AFNI could also be used.

FAST segments tissue according to a hidden Markov random field model and an associated expectation-maximization algorithm, that has been shown to be robust and reliable when compared to finite mixture based models, that can be sensitive to noise (Zhang, Brady et al. 2001). Prior to segmentation with FAST, the structural image should first be brain extracted using the brain extraction tool (BET) (Smith 2002) within FAST. After segmentation, FAST will output partial volume maps for GM, WM and CSF as shown in Figure 5.

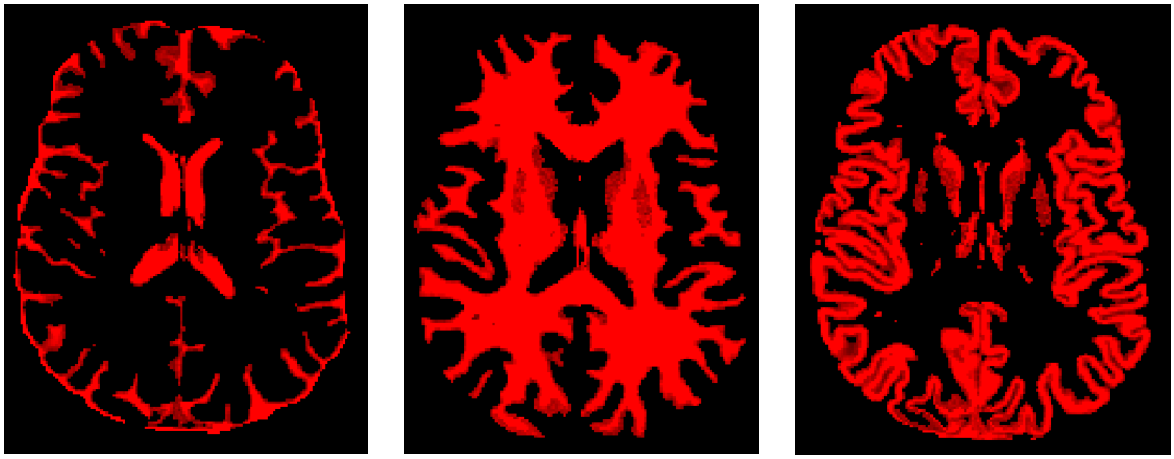


Figure 5 - The results of partial volume segmentation using FAST (parameters described in-text), LHS: CSF mask, MIDDLE: WM mask, RHS: GM mask. CSF: cerebrospinal fluid; WM: white matter, GM: grey matter.

Determination of tissue fractions within SVS voxel

To determine the fractions of WM, GM and CSF within each SVS voxel the voxel mask that has been registered to the anatomical space needs to be overlaid with the partial volume maps created using FAST (shown in Figure 5). Both FSL and FreeSurfer have utilities that will achieve this goal, in FAST the utility is '*fslstats*'. Within FAST stats, the mean of the partial volume map for GM, WM and CSF is determined, using the SVS voxel mask. The commands required to achieve this step are shown below in 'Example commands 1 – Quantifying tissue classes within an SVS voxel'.

Example Commands to quantify tissue classes within SVS voxel:

1. **Extract the brain from the T1-image**

   $ t1image.nii extractedbrain.nii.gz -f 0.3 -g 0

2. **Perform tissue segmentation**

   $ fast -t 1 -n 3 -H 0.1 -I 4 -l 20.0 -o /fast_output_folder /extractedbrain.nii.gz

3. **Determine the grey, white and CSF content of the voxel**

   $ fslstats -t /fast_output/fast_output_pve_0 -k SVS_mask.nii –m

Note: '*fslstats*' (step 3 above) needs to be executed for each tissue type, these masks are located in the *fast_output* folder (see step 3 above) and are named *pve_0*, *pve_1* and *pve_2* (shown in **Error! Reference source not found.** is this o rder) assuming three tissue types have been used.

Example commands 1 – Quantifying tissue classes within an SVS voxel.

Absolute quantification with Partial Volume Correction - 1D spectroscopy

To quantify concentrations of metabolites that are visible on the MR timescale, peak area must first be referenced to a known value. Referencing corrects the measured signal intensity for errors associated with variable RF coil gain and allows comparisons to be made between peak intensities of different metabolites. There are several methods available for metabolite quantification such as: internal

metabolite referencing (typically to tCr or tNAA), internal water signal referencing and external reference solutions (Jansen, Backes et al. 2006). The most popular method for absolute quantification of metabolites using MRS is internal water signal referencing which involves acquiring an unsuppressed water spectrum after the 1D MRS acquisition. It is essential that the unsuppressed water spectrum should be acquired in a manner that is identical to the water suppressed spectrum i.e. same voxel location (Jansen, Backes et al. 2006).

When using water signal referencing, it is assumed that the fully relaxed water signal will be proportional to the number of water moles in the voxel (Gasparovic, Song et al. 2006). Additionally, it is assumed that CSF has no significant contribution to metabolite concentrations. Thus, the absolute metabolite concentration, M, is given by:

Equation 7

$$M = \frac{I_{Met}}{I_{W(GM\&WM)}} \times \frac{2}{\#H_M} \times [H_2O]$$

where I_{Met} is the observed metabolite signal, $I_{W(GM\&WM)}$ is water signal arising from GM and WM, $\#H_m$ is the number of protons contributing to the metabolite peak being quantified (e.g. it is 3 for NAA peak at 2.02ppm), 2 is the number of protons contributing to the water signal and [H2O] is the molar concentration of pure water (55.6 mol/l).

The $I_{W(GM\&WM)}$ term can be derived the from the observed water intensity ($I_{W(obs)}$), accounting for the fact that the water signal arises from multiple compartments and is not fully relaxed:

Equation 8

$$I_{W(obs)} = (f_{GM} \times I_{H2O\_R} \times R_{H2O\_GM}) + (f_{WM} \times I_{H2O\_R} \times R_{H2O\_WM)}) + (f_{CSF} \times I_{H2O\_R} \times R_{H2O\_CSF})$$

where f_X are the water fractions (note not the volume fractions) contained within each tissue class (GM, WM, CSF), I_{H2O_R} refers to the fully relaxed water signal, the relaxation attenuation factors (R_{H2O_X}) are found using Equation 9 and accounts for the fact water relaxes differently depending on which tissue class is it is contained within:

Equation 9

$$R_{H2O\_X} = e^{-\frac{TE}{T2_X}}\left(1 - e^{-\frac{TR}{T1_X}}\right)$$

where TE and TR are the echo and repetition times, respectively, $T1_X$ and $T2_X$ are the T1 and T2 relaxation times of water in tissue compartment X.

Values for T1 and T2 relaxation times of water at 3T can be found in the literature (Wansapura, Holland et al. 1999, Ethofer, Mader et al. 2003) and are shown below in Table 3.

Table 3 - In vivo T1 and T2 water relaxation times (ms) at 3T for relaxation correction using Equation 9.

	GM	WM	CSF
T1	1470	1060	3000
T2	110	74	200

The $I_{W(GM\&WM)}$ is found by rearranging Equation 8 for I_{H2O_R} and subtracting the CSF fraction (f_{CSF}):

Equation 10

$$I_{W(GM\&WM)} = \frac{I_{W(obs)}(1 - f_{CSF})}{(f_{GM} \times R_{H2O\_GM}) + (f_{WM} \times R_{H2O\_WM)}) + (f_{CSF} \times R_{H2O\_CSF})}$$

Finally, Equation 7 and Equation 10 can be combined to yield:

Equation 11

$$M = I_{Met}\left(\frac{(f_{GM}R_{H2O\_GM}) + (f_{WM}R_{H2O\_WM)}) + (f_{CSF}R_{H2O\_CSF})}{I_{W(obs)}(1 - f_{CSF})}\right) \times \frac{2}{\#H_M} \times [H_2O]$$

The fractions of water in different tissue compartments are represented by f_{GM}, f_{WM} and f_{CSF}, which can be expressed in terms of tissue segmentation fractions:

Equation 12

$$f_X = \frac{c_X v_X}{0.82v_{GM} + 0.73v_{WM} + 0.98v_{CSF}}$$

where, c_X is the water content in GM (0.82), WM (0.73) and CSF (0.98) (Allen 1990) and v_X is the tissue volume fraction that is output as a result of SVS voxel segmentation. Ernst et al first showed that tissue compartments could be estimated using bi-exponential fitting of the T2 decay of brain water signal (Ernst, Kreis et al. 1993). However, for many studies estimating the brain tissue water and CSF using

relaxation experiments is not practical due to time limitations. As neuro-imaging software packages have improved, the standard method of estimating the tissue compartments has become structural image segmentation (Wang and Li 1998, Weber-Fahr, Ende et al. 2002, Gasparovic, Song et al. 2006).

A common tool for metabolite quantification is LCModel, where prior knowledge is used to fit a basis set of experimental or simulated spectra to the acquired in vivo spectrum (Provencher 1993). This technique is useful as it allows for the estimation of overlapping resonances such as glutamate and glutamine (Jansen, Backes et al. 2006). Partial volume correction can be applied using LCModel by adjusting the 'WCONC' term according to the equation (Lee, Caparelli et al. 2013, Provencher 2015) :

Equation 13

$$\mathrm{WCONC} = \frac{[\mathrm{H_2O}]\left(\left(\mathrm{f_{GM}R_{H2O\_GM}}\right) + \left(\mathrm{f_{WM}R_{H2O\_WM)}}\right) + \left(\mathrm{f_{CSF}R_{H2O\_CSF}}\right)\right)}{(1 - \mathrm{f_{CSF}})}$$

If the above approach is used, 'WCONC' variable needs to be calculated for each SVS voxel prior to LCModel analysis and input at the time of fitting.

Alternatively, total water concentration in the voxel can be ignored and a correction for CSF can be applied according to (McLean, Woermann et al. 2000):

Equation 14

$$\mathrm{C} = \mathrm{C_0}\left(\frac{1}{1 - \mathrm{v_{csf}}}\right)$$

where C is the corrected metabolite concentration, $\mathrm{C_0}$ is the LCModel output and $\mathrm{v_{csf}}$ is the volume fraction of CSF contained within the SVS voxel. This method only considers the partial volume effect of CSF.

Partial Volume Correction – 2D spectroscopy

Unfortunately, 2D MRS quantification methods are not as mature as those available for 1D MRS. Typically, 2D data is quantified by integration after manual peak picking as described here (Mountford, Quadrelli et al. 2015), however, prior knowledge based fitting algorithms have started to emerge(Schulte and Boesiger 2006). The most common peak quantification method used when analysing 2D spectroscopy is the internal endogenous marker method. When using this technique, a

peak ratio is calculated using an internal reference (diagonal singlet of Cr or NAA for example), the metabolite concentration is then determined by multiplying the peak ratio by the *in-vivo* concentration of the internal reference (Jansen, Backes et al. 2006). However, most commonly the final step is not carried out and just the peak ratio is determined.

It is possible to reference peak volumes to the internal water signal, the main advantage is the water signal is less effected by physiological perturbations than other metabolites. However, this technique still needs to be used cautiously as several studies have found that brain water can change in conditions such as multiple sclerosis (Laule, Vavasour et al. 2004), neoplasia (Grasso, Alafaci et al. 2002) and hydrocephalus (Grasso, Alafaci et al. 2002) (Jansen, Backes et al. 2006).

Metabolite ratios are determined according to:

Equation 15

$$\frac{I_{Met}}{I_{W(GM\&WM)}} = I_{Met}\left(\frac{\left(f_{GM}R_{H2O\_GM}\right) + \left(f_{WM}R_{H2O\_WM)}\right) + \left(f_{CSF}R_{H2O\_CSF}\right)}{I_{W(obs)}(1 - f_{CSF})}\right)$$

where f_{GM}, f_{WM} and f_{CSF} can be determined using Equation 12.

Quantification of other MRI metrics

Being able to quantify other MRI metrics within an SVS voxel can be useful for carrying out correlation or regression analysis. For example, as a part of a multiple sclerosis, study it may be of interest to know the total volume of T2 hyper-intense lesions within the SVS voxel. Alternatively, researchers may like to correlate diffusion metrics such as fractional anisotropy (FA, a measure of the degree of diffusion anisotropy) or mean diffusivity (MD, a measure of apparent diffusion).

We will assume that diffusion data has been corrected for eddy current distortions and a tensor model has been fitted, allowing FA and MD to be calculated. Prior to extracting FA and MD values within the SVS voxel, the diffusion data must be co-registered to the structural image that was used to prescribe the SVS voxel location. This can be achieved using the FAST tool FLIRT (Jenkinson and Smith 2001, Jenkinson, Bannister et al. 2002), specifically the 'epi_reg' script. This script registers diffusion EPI images to structural images, such as T1-weighted images. In the example commands shown below, the b=0 s/mm2 images are registered against the structural images, as they provide better tissue contrast which is important for good quality image registration. Additionally, in this example command 'epi_reg'

will perform EPI distortion correction at the same time as EPI registration. After registering the diffusion tensor images (DTI) to the structural space, the mean values for MD or FA can be determined using the 'fslstats' command. A summary of this process is shown below in the 'Example commands 2' box.

This same process of co-registration, transformation and data extraction, can be used for any other imaging metric where data is prescribed to an imaging voxel e.g. blood oxygenation level dependent (BOLD) imaging. However, if the metric of interest e.g. white matter lesions in a multiple sclerosis study, are already in the structural space then the registration step can be skipped.

Example Commands to quantify DTI metrics within SVS voxel:

1. Co-register diffusion data with SVS prescription structural image

   $ epi_reg --epi=my_hifi_b0 --t1=t1image.nii --t1brain=extractedbrain.nii.gz --out=dwi2struct --fmap my_fieldmap_rads --fmapmag=my_fieldmap_mag --fmapmagbrain my_fieldmap_mag --echospacing=0.000345 --pedir=-y --wmseg/fast_output/fast_output_pve_2

2. Transform the DTI data into the structural space

   $ applywarp --in= FA_map.nii.gz --ref= t1image.nii --out= FA_2_struct --warp= dwi2struct_warp

Example commands 2 - Quantifying other imaging metrics within the SVS voxel.

Example registration and segmentation

A single voxel 1D spectrum was acquired from the hippocampus using a voxel size 3×1.5×1.5cm=6.75cm^3, from a patient who had previously been diagnosed with relapsing and remitting (RRMS) multiple sclerosis. Multiple sclerosis (MS) is neurodegenerative condition characterised by autoimmune mediated demyelination. At the time of scanning (Prisma, Siemens, Erlangen) the following structural images were acquired using a 64 channel head coil: 3D T1-weighted MPRAGE sequence (TR/TE/TI=2000/3.5/1100 ms, flip angle = 7°, field of view= 256x256 mm, voxel size 1x1x1mm3, IPAT 2, acquisition time 4:48 minutes); T2 Fluid-attenuated inversion recovery (FLAIR)

(TR/TE/TI=5000/386/1800 ms, echo train duration: 858ms, field of view = 256 × 256 mm^2, with spatial resolution of 1×1×1 mm^3, IPAT 3 acquisition time 4:12) .

The SVS voxel mask was created according to the steps outlined above in the 'co-registration of voxel mask section'. The results of this step are shown in Figure 4 and can be compared to the original prescription voxel image that was saved at the time of scanning.

In MS evidence of macroscopic tissue abnormalities can be detected on MRI (Filippi and Rocca 2011), using a T2 FLAIR sequence for example. On T2 FLAIR, MS plaques appear hyperintense and thus it is important to know if there is a lesion within the SVS voxel as the spectrum from these areas will be different to normal appearing brain (Oz, Alger et al. 2014). Figure 6 shows an example of a hippocampal SVS voxel that contains white matter lesions. T2 FLAIR lesions were segmented using the lesion growth algorithm (Schmidt, Gaser et al. 2012) as implemented in the LST toolbox version 2.0.6 for SPM.T2 FLAIR lesions were segmented using the lesion growth algorithm (Schmidt, Gaser et al. 2012) as implemented in the LST toolbox version 2.0.6 for SPM. The algorithm first segments the T1 images into the three main tissue classes (CSF, GM and WM). This information is then combined with the co-registered FLAIR intensities in order to calculate lesion belief maps. By thresholding these maps with a pre-chosen initial threshold (κ) an initial binary lesion map was obtained which was subsequently grown along voxels that appear hyperintense in the FLAIR image. The initial κ threshold was selected by iterating κ and performing a visual inspection, for this data a κ value of 0.1 was selected. A binary lesion mask was created for each participant using a threshold of 0.5. Hypointense lesions on the T1 MPRAGE were determined using the binary lesion mask and filled with intensities similar to voxels not contained within a lesion. Lesion filling was performed using the LST toolbox (Schmidt, Gaser et al. 2012) to improve volume measurements and prevent errors in partial volume segmentation (Gelineau-Morel, Tomassini et al. 2012).

Tissue classes were segmented from the lesion filled T1 MPRAGE using FAST (Zhang, Brady et al. 2001) as described above. The tissue class volume fractions within the hippocampal voxel were calculated according to the steps in the 'Tissue classes were segmented from the lesion filled T1 MPRAGE using FAST (Zhang, Brady et al. 2001) as described above. The tissue class volume fractions within the hippocampal voxel were calculated according to the steps in the 'Example commands 1' box above. Finally, the volume fractions and T2 FLAIR lesions were quantified within the hippocampal voxel, the results of this step are shown in Table 4.

Table 4 - Fractions of CSF, GM, WM and WM lesion volume contained within the example SVS voxel

	CSF	GM	WM	WM Lesion (mm3)
Hippocampus	0.029	0.421	0.550	117

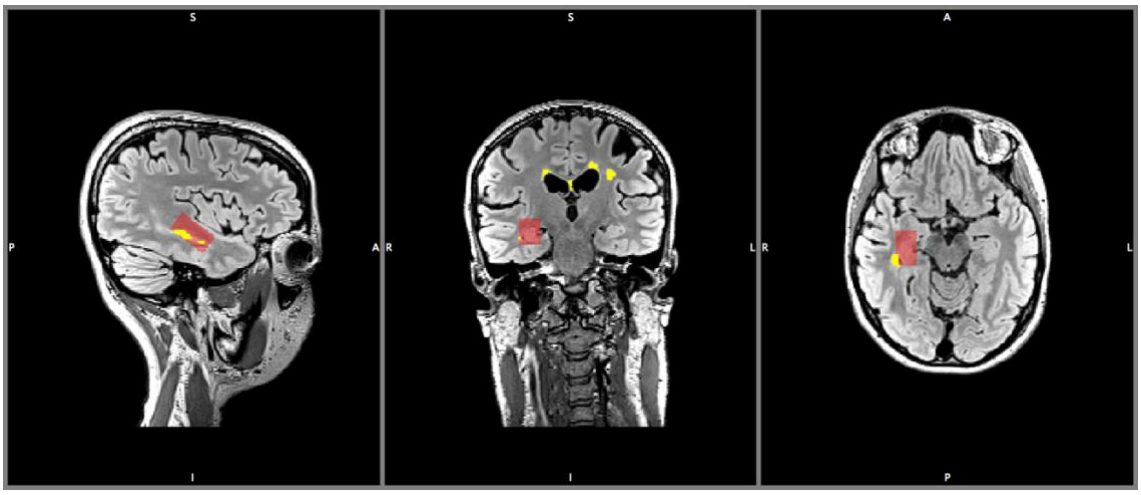


Figure 6 - T1 MPRAGE image of patient with MS, the hippocampal SVS voxel is overlaid in red, shown in yellow is the binary lesion map that was constructed using a FLAIR image. Notice that there are white matter lesions contained within the hippocampal SVS voxel. T1 MPRAGE image was acquired at 3T using a 64 channel head coil: (TR/TE/TI=2000/3.5/1100 ms, flip angle = 7°, field of view= 256x256 mm, voxel size 1x1x1mm3).

Conclusion

Partial volume effects have the potential to introduce error when quantifying metabolites using proton magnetic resonance spectroscopy (^{1}HMRS). Here we have provided a comprehensive outline of how to undertake partial volume correction, using freely available neuro-imaging packages and how these corrections can be applied to 1D and 2D spectroscopy (L-COSY). Additionally, a guide to extracting other MRI metrics of interest (such as FA or MD) from the SVS voxel volume was provided.

Acknowledgements

We thank A/Prof Jeanette Lechner Scott and Dr Karen Ribbons for allowing us to use data acquired from a participant diagnosed with multiple sclerosis.

Chapter 4

Posttraumatic Stress Disorder

Systematic review of *in-vivo* Magnetic Resonance Spectroscopy for the assessment of Post-Traumatic Stress Disorder

Scott Quadrelli, Carolyn Mountford, Saadallah Ramadan

Abstract

Posttraumatic stress disorder (PTSD) is a trauma and stressor-related disorder that results in complex somatic, cognitive, affective and behavioural effects, after exposure to traumatic event(s). Conventional imaging (T1 and T2 weighted magnetic resonance imaging) has little to offer in the way of diagnosis of mental health conditions such as PTSD and there is currently no objective diagnostic test available. Magnetic resonance spectroscopy (MRS) allows for non-invasive measurement of metabolites and neurochemicals in the brain using a conventional MRI scanner and offers the potential to predict, diagnose and monitor PTSD. This systematic review summarises the results of 24 MRS studies, performed between 1998 and 2017, to measure neurochemical differences, occurring as a consequence of PTSD. The most consistent finding in subjects with PTSD is lower N-acetylaspartate levels in the hippocampus and anterior cingulate cortex, with and without atrophic change. More recent studies, using more advanced techniques and modern hardware, have shown evidence of glutamatergic dysfunction and differences in gamma-aminobutyric acid levels in the brain of patients with PTSD. Conflicting results have been reported in choline-containing metabolites and there is emerging evidence of glutathione being affected. Myo-inositol and creatine are unchanged in the majority of studies.

Introduction

Posttraumatic stress disorder (PTSD) is a trauma and stressor-related disorder that results in complex somatic, cognitive, affective and behavioural effects, after exposure to traumatic event(s). PTSD is precipitated by a number of factors, including exposure to actual or threatened death or serious injury or a response to intense fear, helplessness or horror. PTSD is characterised by persistent re-experiencing of the traumatic event; avoidance of stimuli related to the trauma; and hyperarousal for at least one month post trauma (American Psychiatric Association 1994), leading to significant psychosocial impairment for patients. PTSD is common in the general and military populations with an estimated 12-month prevalence of 5.2% vs. 8.3%, respectively (McFarlane, Hodson et al. 2010).

In vivo neuro proton magnetic resonance spectroscopy (MRS) provides a non-invasive measurement of metabolites in the brain using a conventional magnetic resonance imaging (MRI) scanner at a field strength of 1.5 or 3T. In vivo MRS can measure differences in parenchymal metabolism in both the healthy and diseased brain (Mountford, Stanwell et al. 2010) and has the potential to provide biomarkers for psychiatric disease, which often has no characteristic findings on conventional T1 and T2 weighted structural MRI (Foerster, Petrou et al. 2012, Murray, Przybelski et al. 2014). Up to 35 signals from metabolites, lipids, and macro-molecules can be measured using one dimensional (1D) neuro MRS at 3T (Provencher 2001). Commonly used 1D MRS pulse sequences, include Point-RESolved Spectroscopy (PRESS) and Stimulated Echo Acquisition Mode (STEAM). Shown below in Figure 7 is a typical 1D neuro MRS spectrum (3T using a 64-channel head coil) from a healthy human brain labelled with the most commonly measured metabolites. MRS has the potential to shed further information on the pathogenesis and mechanisms behind PTSD, particularly when state of the art scanners are employed, which measure neurotransmitters such as gamma-aminobutyric acid (GABA), aspartate (Asp) and glutamate (Glu) non-invasively. This will further advance knowledge gained from other neuroimaging techniques such as functional magnetic resonance imaging (fMRI) and diffusion-weighted imaging (DWI). Additionally, in vivo neuro MRS is a promising technique that may help to predict patients at risk for PTSD and to non-invasively diagnose and monitor PTSD.

In this review, we synthesize previous work utilising proton MRS to characterise metabolic differences in the brain of patients with PTSD. Also, we give researchers a broader background on key MRS metabolites and brain regions implicated in the pathogenesis of PTSD that will help inform research findings and conclusions. Finally, we aim to determine if certain metabolites correlate with clinical

variables, to identify possible MRS biomarkers of disease severity. We conclude with a discussion on the limitations and outstanding challenges facing researchers, utilising MRS to investigate PTSD.

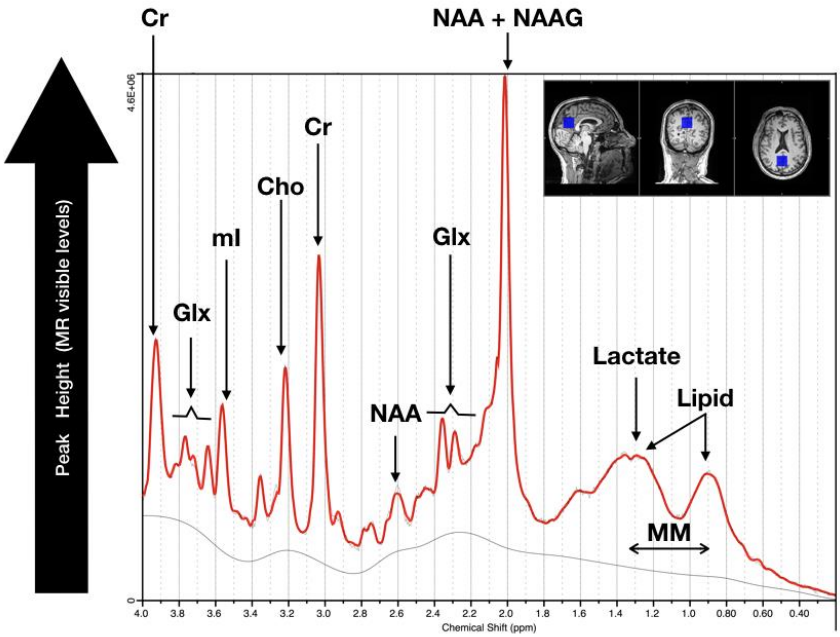


Figure 7 – Example of a MRS spectrum from the posterior cingulate cortex (PCC) of a healthy human brain at 3T, acquired using PRESS (TE = 30ms, TR = 1500ms) with important metabolite resonances labelled. Inset: MRS voxel location in the PCC. Adapted with permission from Neurospectroscopy: The Past, Present and Future. Carolyn E. Mountford, Peter Stanwell, Alexander Lin, Saadallah Ramadan, and Brian Ross. Chemical Reviews 2010 110 (5), 3060-3086. DOI: 10.1021/cr900250y. Copyright (2010) American Chemical Society". Abbreviations: Cr – Creatine; Glx – Glutamine and Glutamate; mI – myo-inositol; Cho – Choline; NAA – N-Acetylaspartate; NAAG - N-actelyaspartylglutamate; MM – macromolecules.

Brain Regions Implicated in PTSD

Multiple brain regions, specifically, the medial prefrontal cortex (mPFC), anterior cingulate cortex (ACC), amygdala, hippocampus and insular cortex have been implicated in the pathogenesis of PTSD from imaging studies using positron emission tomography (PET), fMRI and structural MRI (Etkin and Wager 2007, Lanius and Olff 2017).

Hippocampus

The hippocampus is anatomically and functionally closely related to the amygdala; both structures play a significant role in the fear, fear extinction and anxiety networks, along with the hypothalamic-pituitary axis (Tovote, Fadok et al. 2015), making the hippocampus an ideal region to interrogate with MRS.

Initially, animal models were used to identify a relationship between stress and morphological change of the hippocampus. This damage may be the result of Glu toxicity, prolonged exposure to elevated glucocorticoids or differences in brain derived neurotrophic factors or a combination of all these factors (McEwen, Angulo et al. 1992, Sunanda, Rao et al. 1995, Magarinos, McEwen et al. 1996, Sapolsky 1996, McEwen 2000).

Several volumetric meta-analyses have identified a reduction in hippocampal volume (Kitayama, Vaccarino et al. 2005, Smith 2005). Most recently O'Doherty et al. (2015) reviewed 36 studies quantifying human hippocampal volume and found smaller volumes bilaterally in PTSD subjects, with a greater reduction in the left hippocampus. It is still unclear if hippocampal atrophy is congenital or acquired in PTSD; however, twin studies have provided early evidence that lower hippocampal volume may be a pre-existing risk factor for PTSD (Gilbertson, Shenton et al. 2002). Spectroscopy has the potential to provide complementary, non-invasive metabolic measurement of the MR visible chemistry and neuronal integrity of the hippocampus and may identify these differences prior to structural MRI changes (Schuff, Neylan et al. 2001). Currently, there are no prospective MRS studies that have aimed to determine if metabolic differences precede atrophy in PTSD.

Amygdala

The amygdala appears to play a key role in the pathogenesis and expression of PTSD. Fear conditioning relies on an interplay between the amygdala and the prelimbic cortex of the mPFC (Bauer 2016). The amygdala is responsible for the association of conditioned and unconditioned stimuli and the mPFC is involved in the expression of fear memory (Alden, Besson et al. 1994, Amorapanth, LeDoux et al. 2000). The amygdala is also critical in the extinction of fear memories, thought to occur within the basolateral amygdala (Amano, Duvarci et al. 2011).

Functional MRI studies have identified increased activity within the amygdala in PTSD (Hughes and Shin 2011). However, volumetric differences within the amygdala have been less clear cut; a recent meta-analysis concluded that there was no significant reduction in amygdala volume when trauma exposed controls were compared to PTSD subjects (O'Doherty, Chitty et al. 2015). Yet the same meta-analysis

identified smaller amygdala volumes bilaterally in PTSD subjects when compared to healthy controls (O'Doherty, Chitty et al. 2015).

There are technical challenges of acquiring spectroscopy of the amygdala due to its small size, measuring 1.2cm^3 on average (Brabec, Rulseh et al. 2010). There are currently no MRS studies published on neurochemical differences in the amygdala in PTSD. However, one group has shown it is technically feasible to acquire MRS data from the amygdala (Nacewicz, Angelos et al. 2012). There are now clinical research scanners at 3T that may well allow MRS of this region to be evaluated.

Medial Pre-Frontal Cortex

The mPFC refers to the medial anterior frontal lobe parenchyma, a region with bidirectional white matter connections with the amygdala (Pape and Pare 2010). The mPFC consist of two subregions: the prelimbic cortex (PL) and infralimbic cortex (IL), that in the rodent brain exert dual control over the amygdala, with the IL supporting fear expression and the PL supporting fear extinction. The human mPFC also contains the ACC, as shown in Figure 8. The human dorsal anterior cingulate cortex (dACC) is thought to be a functional homologue of the rodent PL (Milad and Quirk 2012). The IL has been implicated in the expression of learned but not innate fear (Vidal-Gonzalez, Vidal-Gonzalez et al. 2006, Sierra-Mercado, Padilla-Coreano et al. 2011), and in humans the ventromedial pre-frontal cortex (vmPFC) is thought to function as the homologue of the IL (Milad and Quirk 2012). Additionally, the mPFC appears to play a role in the cognitive processing of emotional tasks (Phan, Wager et al. 2004), integrating affective stimuli received via inputs from the amygdala and hippocampus and evaluating these inputs against previous experience and behavioural goals (Lanius and Olff 2017).

Anterior Cingulate Cortex

The cingulate cortex is located in the centre of the brain, anterior to the splenium of the corpus callosum. The anterior component of the cingulate cortex is subdivided into the dorsal, ventral and rostral components and two of these regions are shown in Figure 8. The dACC has been implicated in the expression and acquisition of conditioned fear responses (Milad, Quirk et al. 2007).

Higher resting state fMRI (rs-fMRI) and task-based activity (non-emotional tasks) has been identified in the dACC in PTSD patients and their non-traumatised twins (Shin, Lasko et al. 2009), suggesting that higher rs-fMRI activity within the dACC may predispose for the development of PTSD.

The rostral anterior cingulate cortex (rACC) has also been implicated in the pathogenesis of PTSD, where it has been shown to be hypoactive in fMRI studies, resulting in reduced amygdala inhibition (Shin and Liberzon 2010, Admon, Milad et al. 2013). Like the hippocampus, the ACC has been shown to have

smaller volumes bilaterally in PTSD when compared to controls and these differences are thought to be acquired as a result of the disease (Kitayama, Quinn et al. 2006, Kasai, Yamasue et al. 2008). There is also some evidence that the cortical thickness of the dACC correlates with the magnitude of a conditioned fear response (Hartley, Fischl et al. 2011).

Insula Cortex

The insula cortex is located within the centre of the cerebral hemispheres and is extensively interconnected to regions, including the primary and secondary sensory cortex, anterior cingulate cortex, hippocampus, amygdala and the autonomic nervous system (Augustine 1996). The insula cortex has been associated with interoceptive awareness of negative emotion such as anticipatory anxiety of guilt (Phan, Wager et al. 2004) and has been shown to activate in response to fearful facial expressions (Calder, Lawrence et al. 2001) and fearful conditional stimuli (Sehlmeyer, Schoning et al. 2009). Therefore, the insula cortex is well positioned to convey somatic sensations, displayed as higher autonomic activation, elicited by interoceptive negative emotion. In the meta-analysis undertaken by Etkin and Wager (2007), the insula and amygdala demonstrated higher activation in PTSD, a finding also noted in social anxiety disorder and specific phobia disorders, suggesting a common mechanism. Few studies have investigated volumetric differences in the insula, however, two studies have identified lower gray matter density in PTSD subjects (Kasai, Yamasue et al. 2008, Nardo, Hogberg et al. 2010) and one study performed in twins, suggested the abnormality may be acquired in PTSD.

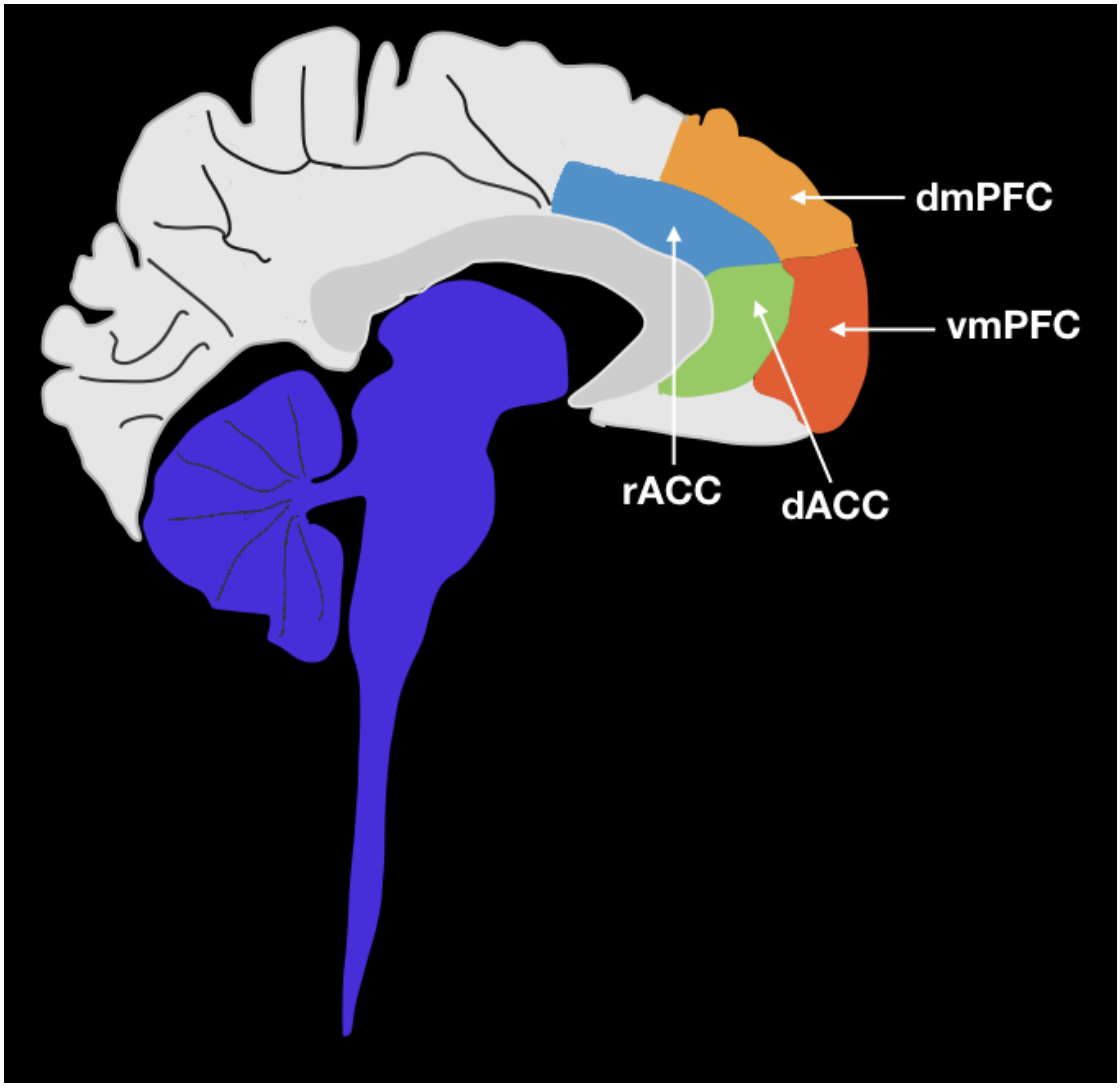


Figure 8 - The medial prefrontal cortex (dmPFC: dorsal medial prefrontal cortex and vmPFC: ventromedial prefrontal cortex) with the anterior cingulate cortex (rACC: rostral anterior cingulate cortex and dACC: dorsal anterior cingulate cortex) are implicated in the pathogenesis of PTSD.

Important brain metabolites

Below, a brief introduction to the metabolites that have been implicated in PTSD is given.

N-Acetylaspartate (NAA)

NAA is an amino acid derivative present in the brain in high concentrations and provides the most intense singlet resonance at 2.01 ppm, in a proton MRS spectrum acquired from a healthy brain (Tallan, Moore et al. 1956, Tallan 1957, Luyten and den Hollander 1986, Moffett, Ross et al. 2007). The

resonance is shouldered by the N-actelyaspartylglutamate (NAAG) singlet peak (Govindaraju, Young et al. 2000).

The function of NAA is an ongoing area of research, however, it is thought to function as an organic osmolyte. As an immediate precursor of NAAG, NAA acts as a source of acetate and facilitates energy metabolism in neuronal mitochondria (Moffett et al. 2007).

Reduced NAA was first thought to indicate an irreversible loss of neuronal density. However, a decrease in NAA has been shown to be reversible in conditions such as acute brain injury or methamphetamine abuse (Maddock and Buonocore 2012) and is thought to represent reversible neuronal or mitochondrial dysfunction (Moffett, Ross et al. 2007). Therefore, the proton MRS signal from NAA indicates the viability, health and density of neurons and therefore can indicate neuronal mitochondria dysfunction (Moffett, Ross et al. 2007).

Glutamate and Glutamine

Glutamate functions mainly as an excitatory neurotransmitter and is the most abundant amino acid in the human brain (6-13mmol kg_{ww}^{-1}) (Govindaraju, Young et al. 2000, Ramadan, Lin et al. 2013). The resonances of Glu and Gln are in the region of 3.74 – 3.75 ppm and 2.04-2.45 ppm and are overlapped by Gln, GABA and NAA (Govindaraju, Young et al. 2000). The chemical structures of Glu and Gln are very similar resulting in significant overlap in the spectra collected at 1.5, 3T and 4T but not at 7T (Terpstra, Cheong et al. 2016), and for this reason the composite resonances are referred to as Glx at lower field strengths.

Gln is a precursor for the excitatory neurotransmitter Glu and is located within astrocytes (Govindaraju, Young et al. 2000, Lin, Ramadan et al. 2015). The primary role of Gln is to act as the 'storage form' of amino acid neurotransmitters such as Glu and GABA.

GABA

GABA is the primary inhibitory neurotransmitter in the brain. It contains three methylene (CH_2) groups that produce a complex one-dimensional spectrum with resonance multiplets centred at 1.89, 2.28 and 3.01 ppm. There is considerable overlap with resonances of NAA, Glu and creatine (Cr), which makes GABA difficult to reliably distinguish using conventional one-dimensional spectroscopy at 1.5 and 3T field strengths (Maddock and Buonocore 2012). It is important to remember that in a one-dimensional MR spectrum the resonances are overlapping so the resultant difference may be due to other contributing molecules. Specialised pulse sequences are regarded as accurate ways to quantify GABA,

the most common of which is MEGA-PRESS J–difference editing sequence (Mescher, Merkle et al. 1998) and 2D JPRESS (Jensen, Licata et al. 2009). Alternatively, GABA can be measured in conventional short echo time (TE) MRS using a fitting algorithm, such as the one implemented in LCModel (Provencher 1993).

Myo-inositol (mI)

Myo-inositol is the most abundant form of inositol found in the brain. Using proton MRS, mI has a prominent mutiplet peak at 3.52 and 3.61 ppm (Govindaraju, Young et al. 2000). It is thought to be a astroglial marker (Brand, Richter-Landsberg et al. 1993).

Choline (Cho)

Choline is a vital constituent of many phosphoglycolipids, present within cell membranes (Koolman and Röhm 2013), and contains contributions from glycerophosphorylcholine and phosphorylcholine. An increase in glycerophosphorylcholine and/or phosphorylcholine can indicate an increase in synthesis or breakdown of membrane phospholipids and results in a subsequent increase in the 3.21 ppm peak on proton MRS (Geddes, Panchalingam et al. 1997, Boulanger, Labelle et al. 2000).

Total Creatine (tCr)

Total Cr gives rise to the second largest peak in a typical brain MR spectrum at 3.03 ppm and contains contributions from Cr and phosphocreatine (PCr). PCr is a precursor for ATP and therefore tCr is thought of as an in vivo mitochondrial energy marker. tCr has been shown to be relatively stable in the healthy brain, with no significant daily intrasubject variation and for that reason is often used as an internal reference for other metabolites (Saunders, Howe et al. 1999, Soreni, Noseworthy et al. 2006). However, tCr been shown to be disturbed in pathologies that alter cell metabolism, such as stroke and malignancy (Mathews, Barker et al. 1995, Howe, Barton et al. 2003) and is therefore used with caution as an internal reference metabolite.

Method

Literature search and inclusion criteria

A literature search was conducted using PubMed and PsychNet in April 2018 using the following keywords: (i) “post-traumatic stress disorder” and (ii) “PTSD” combined with the following subcategory keywords: (iii) “magnetic resonance spectroscopy” and (iv) “MRS”. Studies were selected if they satisfied the following conditions: in vivo human MRS studies, the patient group had a Diagnostic and Statistical Manual (DSM) based diagnosis of PTSD, were written in English and were compared with

either a healthy or traumatised control group that did not have PTSD. None of the studies included participants with severe traumatic brain injury. Where any two or more studies included the same or overlapping patient populations, only the study with the largest sample size was included.

Results

A total of 27 studies were identified. Three studies were excluded: the study by De Bellis et al. (2001) was excluded as it was a case study with a single participant; the investigation performed by Henigsberg et al. (2011) as it was a therapeutic study and Neylan et al.'s (Neylan, Schuff et al. 2003) study as it contained overlapping populations with Schuff et al.'s (Schuff, Neylan et al. 2001) study. The remaining 24 studies were published between 1998 and 2017 and are summarised below in Table 5. Metabolic findings relevant to PTSD are shown below.

N-Acetylaspartate

The most consistent MRS finding identified in PTSD was a reduction in NAA. Fifteen studies investigated NAA levels in the hippocampus. Several early studies refer to the to the hippocampus as the medial temporal, as the MRS voxel contained the hippocampus medial temporal lobe parenchyma. Six studies used absolute quantification (studies 3, 5, 13, 14, 16, and 24) and half of these found no significant difference in NAA (studies 5, 14 and 16). The positive studies found a statistically significant reduction in NAA levels in both hippocampi. Study 5 found a trend toward lower NAA in the hippocampus ($p = 0.054$), however, the sample was small which may have limited its statistical power. Eleven studies (1, 4, 6, 8, 9, 11, 12, 13, 14, 15 and 17) used Cr as an internal reference (some used both absolute and relative quantification). Four studies (6, 8, 12 and 13) identified no difference in the hippocampal NAA/Cr. One study (1) found higher NAA/Cr in the right hippocampus only, two studies (4 and 11) found lower NAA/Cr in the left hippocampus only and the remaining studies (9, 14, 15 and 17) found a significant reduction in NAA/Cr in both hippocampi.

Several early studies hypothesised that reductions in NAA may precede morphological change in the hippocampus. Many studies (3, 9, 12, 14, 16, and 24) found no significant difference in hippocampal volume, while the majority of studies (1, 4, 5, 6, 8, 12, 13 and 15) did not quantify the hippocampal volume. Shu et al. (2013a) identified a significant reduction in the normalised left hippocampus along with a significant reduction in NAA/Cr and Li et al. (2006) found lower NAA/Cr was correlated with lower gray matter density in the left hippocampus.

Eleven studies (2, 9, 10, 13, 14, 15, 18, 19, 20, 21 and 22) investigated NAA levels within the ACC. Several earlier studies (2, 10, 13, 14 and 15) did not specify the exact voxel location within the ACC, two of these studies (13 and 14) identified lower absolute NAA, and two studies found lower NAA/Cr (2 and 15). One study (9) reported lower NAA/Cr in the dACC when PTSD was compared to healthy controls and one other (19) reported no difference in NAA in the dACC, noting that study 19 compared PTSD to trauma exposed controls. One study in the rACC (21) identified lower absolute NAA, while the remaining three studies (19, 20 and 22) investigating this region found no significant difference in NAA. No studies have explored the longitudinal change in metabolites in the ACC with PTSD.

NAA was found to be lower in several other regions of the brain, including the left basal ganglia (Lim, Suh et al. 2003) and the parietal occipital gray matter (Meyerhoff, Mon et al. 2014). Otherwise, no significant difference in NAA was identified in the periventricular white matter (WM) (Lim, Suh et al. 2003), occipital gray matter and white matter (Villarreal, Petropoulos et al. 2002, Seedat, Videen et al. 2005), right insula (Rosso, Weiner et al. 2014), dorsolateral pre-frontal cortex (DLPFC) (Michels, Schulte-Vels et al. 2014), posterior occipital cortex (POC) and temporal lobe (Meyerhoff, Mon et al. 2014, Pennington, Abe et al. 2014).

Glutamate and Glutamine

It is possible that glutamatergic dysfunction reported in several brain regions plays a role in the pathogenesis of PTSD, however findings were mixed. It is worth noting that the accurate measurement of Glu and Gln as separate entities is currently only possible at the higher field strength of 7T (Terpstra, Cheong et al. 2016). Six studies (19, 20, 21, 22, 23 and 24) investigated Glx levels in multiple brain regions. In the temporal lobe two studies (19 and 21) identified a reduction in Glx and Glu respectively. Furthermore, a study performed by Pennington et al. (2014) identified higher absolute Glx in the temporal lobe in PTSD participants with co-morbid alcohol use disorder (AUD) when compared to PTSD participants without AUD. Only one study (22) identified a reduction in Glx within rACC using 1D MRS; where the authors noted that Glx was lowest in PTSD subjects followed by those in PTSD remission. Other investigators reported no difference in Glx in the ACC (studies: 19, 20, 21 and 23). More recently Rosso et al. (2017) quantified Glu and Gln in the hippocampus, using a novel TE averaging sequence 2D JPRESS; they found higher absolute Glu and Glu/NAA in the right hippocampus. Statistically significant absolute Glu concentration was positively correlated with re-experiencing symptoms and trauma dose was significantly positively correlated with right hippocampal NAA/Cr. No differences in Glu or Gln were identified in the POC or DLPFC (Meyerhoff, Mon et al. 2014, Michels, Schulte-Vels et al. 2014, Pennington, Abe et al. 2014).

GABA

GABA was investigated in four studies (18, 19, 20 and 21). No difference in GABA/Cr was reported in the dACC (study 18), however, one study (20) identified higher GABA/Cr in the rACC and DLPFC. Lower GABA/Cr was noted within the insula in a single study (18) and lower absolute GABA in the POC and medial temporal lobe (study 21). One study (19) found no difference in absolute GABA in the POC when PTSD participants were compared to healthy controls, however, they did find higher GABA when PTSD sufferers with AUD were compared to PTSD participants without AUD. All the studies described here used MEGA-PRESS to quantify GABA within the brain.

Myo-Inositol

Only two studies (10 and 19) identified differences in mI in PTSD. Seedat et al. (2005) found higher mI/Cr in the ACC of participants who had PTSD secondary to intimate partner violence. No other studies noted mI to be raised in the ACC. In a single study performed in PTSD patients with AUD, absolute mI was lower in the ACC when compared to trauma exposed controls (Pennington, Abe et al. 2014). Multiple studies (7, 13, 21 and 22) found no difference in mI in patients with PTSD, irrespective of brain region.

Choline

Twelve studies (1, 2, 7, 9, 10, 12, 13, 15, 17, 19, 21 and 22) have measured Cho containing compounds in PTSD. The majority of studies (2, 9, 13, 21 and 22) found no difference in Cho containing compounds in the ACC and hippocampus (12, 13, 15, and 17). Conflicting results were reported by two studies (10 and 15) measuring Cho in the ACC using 1D MRS Seedat et al. (2005) found higher Cho/Cr in PTSD when compared to healthy and trauma exposed controls, while Guo et al. (2012) identified lower Cho/Cr in the ACC in PTSD compared to healthy controls in a large cohort. Conflicting results were also reported in the hippocampus (studies 1 and 9). Lower absolute Cho was found in the temporal lobe of participants with PTSD and AUD when compared to PTSD participants without AUD and trauma exposed controls (Seedat, Videen et al. 2005, Pennington, Abe et al. 2014).

Glutathione

One study (20) identified an increase in glutathione/Cr in the ACC and DLPFC in PTSD when compared to trauma exposed controls.

Total Creatine

Two early studies identified a reduction in absolute Cr in the hippocampus and occipital white matter (studies: 2 and 3). Four subsequent studies have identified no difference in absolute Cr concentration (studies 13, 18, 19 and, 21).

Metabolic correlates

Increased hippocampal exposure to glucocorticoids has been proposed as a cause of atrophy in PTSD. To explore the glucocorticoid hypothesis further, two studies correlated serum cortisol levels with hippocampal neuro-metabolites and found differing results. Shu et al. (2013b) identified a negative correlation between serum cortisol and hippocampal NAA/Cr levels, whilst Neylan et al. (2003) (not listed in Table 1 due to overlap with Schuff et al.'s (Schuff, Neylan et al. 2001) population) found a positive correlation between the two.

Two studies have identified correlations with GABA and clinical outcomes in PTSD. Meyerhoff et al. (2014) noted that lower levels of GABA and higher Glu correlated with a higher insomnia severity index. In a small group of participants, Rosso et al. (2014) found lower levels of GABA were correlated with higher state-trait anxiety levels. However, they found no correlation between GABA and severity of symptoms (Rosso, Weiner et al. 2014).

Other reports identified a correlation with re-experiencing symptoms and NAA levels. Ham et al. (2017) identified lower levels of NAA in the ACC were correlated with re-experiencing symptoms and more recently, Rosso et al. (2017) found the same in the hippocampi of PTSD subjects. Shu et al. (2013b) noted a correlation between lower NAA/Cr levels in the hippocampus and higher total clinical administered PTSD scores (CAPs). Otherwise, no significant correlation between NAA and symptom severity was identified (Lim, Suh et al. 2003, Kimbrell, Leulf et al. 2005, Rosso, Crowley et al. 2017).

A positive correlation between absolute Glu levels in the right hippocampus and re-experiencing symptoms was identified by Rosso et al (Rosso, Crowley et al. 2017). Additionally, Harnett et al. (Harnett, Wood et al. 2017) found a positive relationship between dACC and absolute Glx levels and current and future stress disorder symptoms.

A positive correlation was found between disease duration and the anti-oxidant glutathione levels (Michels, Schulte-Vels et al. 2014).

Confounding variables in PTSD research

Current or past alcohol dependence is a common co-morbidity seen in PTSD that has been shown to result in significant differences when PTSD participants with and without AUD are compared (Pennington, Abe et al. 2014) and this is likely to be particularly important for GABA, NAA and Glx (Behar, Rothman et al. 1999).

Multiple studies have controlled or adjusted for the effects of depression and medications (mainly serotonin re-uptake inhibitors) using a linear regression and have found no significant effect (Lim, Suh et al. 2003, Kimbrell, Leulf et al. 2005, Eckart, Kaufmann et al. 2012, Michels, Schulte-Vels et al. 2014). In addition, no significant reduction in NAA has been reported in the medial temporal lobe / hippocampus or hippocampi in depression (Rao, Venkatasubramanian et al. 2011).

Many studies have specifically excluded participants with traumatic brain injury (TBI), given that these individuals may have a higher risk of PTSD, which can be related to the TBI or to a separate event (Bryant 2001, Kennedy, Jaffee et al. 2007, King 2008). None of the studies identified directly compared TBI and PTSD groups.

Two studies compared PTSD subjects to traumatised and non-traumatised control groups (Seedat, Videen et al. 2005, Eckart, Kaufmann et al. 2012), in an effort to determine if an observed effect was due to PTSD or simply trauma exposure. These studies have had mixed results; Seedat et al. (2005) found a significant difference between PTSD and trauma exposed control groups, in the ACC, suggesting that the observed metabolic difference was due to PTSD itself. On the other hand, Eckart et al. (2012) found no significant difference between the PTSD, healthy / trauma exposed control groups using bilateral hippocampal and insula voxels.

Discussion

The current systematic review has summarised findings from 24 proton MRS studies comparing PTSD subjects to controls subjects. There is emerging evidence of neurochemical abnormalities in patients with PTSD, identified using in vivo proton MRS of the human brain. It is important to note that the hardware and post-processing capabilities for in vivo neuro MRS have been steadily improving as early studies were restricted in what they were able to record.

The most consistent findings to date are reductions in NAA in the region of the hippocampus and ACC. Multiple studies have found reductions in both absolute NAA and NAA/Cr in both hippocampi in PTSD participants when compared to healthy and trauma exposed controls. A meta-analysis of MRS studies

performed by Karl et al. (2010) identified lower NAA in the left hippocampus when compared to healthy and trauma exposed controls and lower right hippocampal NAA only when compared to trauma exposed controls. Two possible aetiologies are explained for the laterality of lower hippocampal NAA: the left hippocampus has a larger volume in right handed individuals and perhaps atrophies faster and the left hippocampus has a greater role in declarative memory (encoding and retrieving tasks) and declarative memory is known to be impaired in PTSD (Li, Chen et al. 2006). Since publication of Karl and Werner's meta-analysis (Karl and Werner 2010), there have been three additional studies published that have identified lower NAA/Cr (Guo, Chen et al. 2012, Shu, Xue et al. 2013) and lower total NAA (Rosso, Crowley et al. 2017) in both hippocampi of PTSD participants. NAA can be reduced in the brain for multiple reasons (see '*1.2 Important Brain Metabolites*' above), one of which is lower neuronal density, ultimately leading to morphologic change or atrophy. The findings of lower NAA within both hippocampi correlates with the meta-analyses of structural imaging studies (Kitayama, Vaccarino et al. 2005, Smith 2005). It is still not clear from the data published to date if the MRS differences (lower NAA) precedes morphological changes, however, this would appear very likely, given that multiple studies have identified lower hippocampal NAA without atrophic change. It is worth noting that a large number of studies identified no difference in hippocampal NAA despite the fact that almost all of these studies were performed on veterans and participants with long standing chronic PTSD. This may suggest that the reduction in hippocampal NAA is reversible in PTSD, as has been shown in conditions such as acute brain injury or methamphetamine abuse (Maddock and Buonocore 2012), which is thought to represent reversible neuronal or mitochondrial dysfunction (Moffett, Ross et al. 2007) as described in the introduction. Additionally, the majority of the studies that found lower NAA in the hippocampi of those with PTSD compared to healthy controls, suggesting that the difference is not due to the choice of control cohort (trauma exposed vs. healthy control), as proposed by Karl et al. (Karl, Schaefer et al. 2006).

A smaller number of studies have reported a reduction in NAA within the ACC with increased heterogeneity of results when compared to the hippocampus. However, many of the early studies did not specify the region of the ACC being measured. Studies that have reported NAA in the rACC have given mixed results, some with lower NAA and others with no difference. Differences in the rACC in PTSD are thought to be acquired and the disparity in the MRS results may be due variations in metabolite concentrations over time (Admon, Milad et al. 2013).

More recently, with advancing spectroscopic techniques, researchers have identified glutamatergic dysfunction and differences to GABA in some of the brain regions where we would expect

hypoactivation, based on previous fMRI work, such as the occipital cortex and rACC. In a study of adolescents exposed to earthquake, Yang et al. (Yang, Quan et al. 2015) found lower Glx/Cr in the rACC, in both PTSD and remitted PTSD. However, patients with PTSD had further lower rACC Glx/Cr when compared to remitted PTSD patients, suggesting lower rACC excitatory neurotransmission and therefore lower amygdala inhibition. However, this finding was not supported by Meyerhoff et al. (Meyerhoff, Mon et al. 2014), which may be due the fluctuation of metabolic differences over time in this region. On the other hand, higher Glx has been identified in the rACC of patients with PTSD and comorbid AUD when compared to PTSD patients without AUD (Pennington, Abe et al. 2014) and with an associated increase in GABA in the same region (Meyerhoff, Mon et al. 2014), raising the possibility that alcohol reverses some of the differences described above and partly explains the association of PSTD and AUD. Higher GABA/Cr has been noted in the rACC.

One hypothesis for higher GABA is overexpression of Glu or Gln decarboxylase and/or lower clearance of GABA from the synaptic cleft (Michels, Schulte-Vels et al. 2014), which supports the hypothesis of rACC hypoactivation, resulting in amygdala hyperactivation (Admon, Milad et al. 2013). More recently, higher absolute Glu was identified in the right hippocampus (Rosso, Crowley et al. 2017) raising the possibility that reductions in volume and NAA within the hippocampus may be secondary to excitotoxicity.

No difference in GABA or Glx have been identified in the dACC, noting that only a small number of studies have evaluated this region. fMRI studies have shown this region to be hyperactive in PTSD and the region may be congenitally abnormal in those who have a risk of developing PTSD. Future studies aiming to predict those at risk of developing PTSD may be able to further explore this region.

A small number of studies have reported altered Cho levels in PTSD, but the results are inconsistent. The two studies that identified differences in Cho used relative measures and compared PTSD to healthy controls with conflicting results. One study found a reduction in absolute Cho in the ACC in a population with comorbid AUD (Pennington, Abe et al. 2014). Alterations in Cho may be due to differences relating to glial cells (Seedat, Videen et al. 2005), white-matter or could reflect neuroinflammation (Rohleder and Karl 2006). More work is needed to determine the directionality of differences in Cho containing compounds.

Only one study investigated metabolic differences in the insula cortex, finding lower GABA/Cr with a significant negative correlation with state and trait anxiety. This finding correlates with previous fMRI

studies that have identified increased insula cortex activation in PTSD, social anxiety and specific phobia disorders (Etkin and Wager 2007).

Increasing glutathione correlated with disease duration (Michels, Schulte-Vels et al. 2014). Glutathione is an antioxidant that reduces reactive oxygen species, which can have a detrimental effect at a cellular level. It is not yet known if higher glutathione is a predisposing factor or acquired as the result of PTSD, however, mouse models have also shown overexpression of the genes glyoxalase 1 and glutathione reductase 1 results in higher anxiety, linking the glutathione cycle with anxiety behaviour in a mouse model (Hovatta, Tennant et al. 2005).

Limitations

A challenge for all neuroimaging studies exploring PTSD is the heterogeneity of patient populations. PTSD is known to have multiple comorbidities such as Axis I and II disorders, including depression; alcohol and substance misuse and chronic pain (Scioli-Salter, Forman et al. 2015). Additionally, the majority of patients with PTSD are taking medications, a factor that may confound interpretation of neurochemical differences (Lanius 2010). These factors can make generalising the study results and determining unique metabolic differences in PTSD problematic. There is evidence that AUD alters brain metabolites (Schuff, Neylan et al. 2008) and these participants with AUD should be excluded. Depression has not been shown to contribute to metabolic differences recorded in PTSD and co-morbid depression is so common in this group one could argue that excluding those with depression would result in an unrepresentative sample. Medications such as benzodiazepines and antipsychotics are highly likely to alter neuro-metabolites and should be controlled for. It is less clear if serotonin re-uptake inhibitors are used, but to date no significant difference has been identified in participants using these drugs (see ‘Confounding variables in PTSD research‘ above). A limited number of studies have identified differences between PTSD, healthy control and trauma exposed groups and to ensure that the differences being identified can be attributed to PTSD alone most studies use a trauma exposed control group.

Many of the early spectroscopic studies (55%) utilised a low field strength (1.5T), to measure metabolic differences in patients diagnosed with PTSD. There are inherent limitations in performing neurospectroscopy at low field, such as increased peak overlap; limiting the number of measurable metabolites. Additionally, many of the early studies used peak area or peak fitting with a limited basis set to quantify a limited number of metabolites, such as NAA, Cr and Cho, a technique that has now

been superseded by peak fitting with more extensive basis sets and bioinformatics evaluation of the data point by point (Stanwell P, Siddall P et al. 2010).

Several early studies used a larger MRS voxel within the medial temporal lobe that included the hippocampus. The benefit of this approach is that signal to noise ratio is higher, the limitation is the spectrum contains contributions from surrounding medial temporal lobe parenchyma. As MRI hardware and techniques have improved, researchers have been able to reduce the MRS voxel size, whilst maintaining adequate signal-to-noise and improving the anatomical specificity of the MRS voxel. This difference in voxel volume and anatomic location, may also go some of the way to explaining the variability in MRS findings in the hippocampus.

Finally, there is a large degree of methodological heterogeneity within studies due to the differing MRS pulse sequences available, such as PRESS and STEAM for 1D and the availability of 2D magnetic resonance spectral imaging (MRSI). To further complicate comparisons there are multiple ways in which data can be post-processed, evaluated and corrected. For example, fitted 1D MRS metabolite peaks can be referenced to an internal resonance such as Cr or referenced to tissue water concentration, known as absolute quantification. Some perform partial volume corrections. However, the spectroscopy community have recognised this limitation and recommended protocols for common use (Oz, Alger et al. 2014).

Conclusion and Future directions

The most consistent MRS findings in PTSD is lower NAA in the hippocampi and ACC. The reduced NAA in these regions may be due to neuronal loss and early evidence suggests that reductions in NAA may proceed morphologic change. On the other hand, multiple studies have demonstrated no difference in NAA, raising the possibility that reductions in NAA in PTSD may be reversible or fluctuate over time. Levels of NAA in the ACC and hippocampus have been found to correlate with clinical symptoms by some. There is emerging evidence of glutamatergic dysfunction in the hippocampus, occipital cortex and rACC and it is possible that higher levels of Glu in the hippocampus may be contributing to neuronal loss, secondary to excitotoxicity. Mixed differences have been found in GABA, which is not unexpected due to the dynamic nature of this metabolite. The majority of studies have found no change in mI or Cr, however caution is recommended when using Cr as an internal reference. Further work is required to determine the magnitude and direction of change in Cho containing compounds in PTSD, which to date appear to be reduced or unchanged. A single study has measured glutathione, suggesting an increase in PTSD. MRS studies have been able to identify metabolic changes in the brains of patients

with PTSD, a condition that has no conventional imaging findings. Ongoing work is required to further characterise these differences, whilst attempting to maximising the generalisability of results.

Many of the early spectroscopic studies were limited by hardware. In the last decade, there have been multiple technological leaps in MR scanner technology, shimming and acquisition protocols (Oz, Alger et al. 2014). Higher magnetic field strengths allow for higher signal to noise and greater peak separation, improving comparative accuracy. The newer hardware has made techniques such as 2D and 3D MRSI, highly relevant to the study of PTSD. High spatial resolution 3D MRSI, performed at high field strengths (3 and 7T), will allow for measurement of metabolites in brain regions such as the amygdala (a region that it yet to be examined due to its small volume), insular cortex and the hippocampus. Additionally, k-space under sampling can be utilised to accelerate the acquisition of 3D MRSI, lowering acquisition times. Two-dimensional MR spectroscopy is a technique that allows overlapping and composite metabolic peaks to be deconvoluted (Thomas, Hattori et al. 2003, Ramadan, Andronesi et al. 2011); it has been now applied to several pathologies and offers further metabolic insights not available using 1D MRS alone (Ramadan, Andronesi et al. 2011, Lin, Ramadan et al. 2015, Ramadan, Arm et al. 2015). However, it is yet to be applied to the condition of PTSD and may allow identification of novel biomarkers that have not been identified using 1D MRS.

Another promising technique, which is yet to be applied to PTSD is functional MRS (fMRS). fMRS is a technique that characterises the regional and temporal change of neurotransmitters whilst subjects are exposed to a stimulus, as in fMRI which measures differences in blood oxygen level. This technique is highly relevant to PTSD and may yield further insights into the pathogenesis of the condition. For example, fMRS could be applied to the hippocampus, using a learning task, in participants with PTSD to further evaluate differences in glutamatergic modulation within the hippocampus. Finally, fMRS may improve MRS reproducibility, by controlling for behaviour, potentially reducing the variability of studies quantifying neurotransmitters.

Machine learning techniques are providing a more sophisticated and tailored means of evaluation of the complex, but rich data available from in vivo 1D and 2D neuro MRS, as well as the evaluation of multimodal MRI data. Machine learning techniques may improve the specificity and sensitivity for evaluation of the PTSD condition when using MRS. Machine learning techniques have been used in the past for the analysis of 1D MRS evaluating chronic pain and primary brain lesions (Stanwell P, Siddall P et al. 2010, Ranjith, Parvathy et al. 2015), increasing the specificity of MRS for detecting these conditions, but as yet have not been applied to PTSD.

Tables

Study	Voxel Location/ Size	Field / Sequence / TR (s) / TE (ms) / averages	Participants	Exclusion Criteria	Trauma	Psychological Assessment	Mean CAPS	Findings	Comments
1 (Freeman, Cardwell et al. 1998)	Hippocampi / 20 x 20 x 30 mm^3	1.5T / STEAM / 2 / 30 / 128	21 PTSD subjects 8 veteran controls.	Head injury Dementia Cognitive impairment.	Combat related trauma	CAPS SCID	NG	Right hippocampus: ↓ NAA/Cr ratio Left hippocampus: ↓ Cho/Cr	Did not control for alcohol, substance dependence or handedness. Comorbid major depression. Matched for age and education. No PV correction. SPARC workstation for calculation of peak ratios.
2 (De Bellis, Keshavan et al. 2000)	ACC / 20 x 15 x 10 mm^3	1.5 / STEAM / 1.5 / NG / 150	Children and Adolescents 11 PTSD subjects. 11 Healthy controls. Matched for age, sex and IQ. 2 subjects left handed.	Lifetime exposure to psychotropic drugs A significant medical or neurological illness or history of head injury Gross obesity or growth failure Full scale IQ lower than 80 Presence of DSM-IV anorexia nervosa, autism, substance use disorder, or schizophrenia History of maltreatment of axis I disorder in controls.	Sexual abuse Physical abuse Witnessing domestic violence.	K-SADS-PL Wechsler Intelligence Scale for Children.	NG	ACC: ↓ NAA/Cr ratio. No difference in Cho/Cr.	NAA/Cr calculated with LC-Model.
3 (Schuff, Neylan et al. 2001)	Hippocampi / 210 x 210 x 15 mm^3	1.5T /PRESS CSI / 1.8 / 135 / NG	18 male PTSD. 19 male HC.	Alcohol dependence in the last 5 years Illicit drugs prior 5 years LOC after head trauma Major depression last 3 months Antipsychotics last 6 months.	Combat Veterans	SCID (DSM-IV) CAPS	63	Hippocampi: ↓NAA and Cr in both. Absence of hippocampal atrophy.	Absolute (arbitrary units). Quantified using in house software. Partial volume corrected Controlled for alcohol dependence (5yrs) prior) and found no hippocampal atrophy.
4 (Mohanakrishnan Menon, Nasrallah et al. 2003)	Hippocampi / 15 x 15 x 15 mm^3	1.5T / PRESS / 3 / 30 / 128	14 PTSD. 7 controls (Veterans).	Seizure disorder > 100mg/day Gabapentin Severe head injury with LOC Uncontrolled diabetes Chronic hypertension Chronic alcohol or substance abuse Stroke.	Vietnam veterans. Two participants with non-combat trauma.	Diagnosis made according to the DSM IV. PCL	CAPS not recorded.	Left Hippocampus: ↓ NAA/Cr ratio Right hippocampus: no sig difference in NAA/Cr. No clinical correlations performed.	tCr used as the internal ref. Hippocampal volume was not calculated. Not controlled for handedness, medications (other than gabapentin), recent alcohol or substance abuse. No regression.
5 (Villarreal, Petropoulos et al. 2002)	Hippocampi / 15.3 x 20.3 x 30 mm^3 Bilateral occipital WM / 20 x 20 x 21 mm^3	1.5T / PRESS / 2 / 40 / 128 1.5T / STEAM / 2 / 30 / 128	8 PTSD. 5 Healthy controls.	Major medical or psychiatric diagnosis Alcohol or substance dependence History of head trauma with LOC Seizures Neurological Disorder.	Mixed. Including child sexual assault	SCID-P (DSM-IV) CAPS Beck Depression Inventory Beck Anxiety Inventory.	>60	Left hippocampus: Trend to ↓ NAA (p =0.054). Occipital WM: ↓ Cr no difference in NAA.	Absolute (arbitrary units). Quantified using in house software. Hippocampal volume not quantified.

									No clinical correlates performed. Comorbid depression with medication use in some participants, including benzodiazepines. Matched for handedness.
6 (Brown, Freeman et al. 2003)	Right and left medial temporal lobes / 20 x 20 x 20 mm^3	1.5T / STEAM / 2 /30 / 128	9 POW + PTSD. 12 POW – PTSD.	No history of TBI with LOC No neurological impairment Degenerative neurological condition Dementia. No participants met criteria for current or lifetime alcohol dependence.	Former prisoners of war, with combat experience.	CAPS-2 SCID (DSM-IV)	72	No significant difference between groups. Left MTL: Trend toward ↓ NAA/Cr. Negative correlation between NAA/Cr in the MTL bilaterally and CAPS-2 re-experiencing symptoms.	tCr used as internal reference. Controlled for alcohol, handedness, alcohol dependence. Brain volumes not quantified.
7 (Lim, Suh et al. 2003)	Left basal ganglia, right frontal periventricular WM and right parietal periventricular WM / 20 x 20 x 20 mm^3	1.5T / STEAM / 3 / 30 / 36 / (mixing time 13.7ms)	16 PTSD (10M:6F). 8 Healthy controls.	Organic mental, psychotic, bipolar, psychotic or neurological disorders. Major head injury (LOC >10min) Alcohol or other substance dependence in the last year No psychotropic medications for the previous 4 weeks.	Fire - Public cafe in Korea 1999	SCID -RV	61	Left basal ganglia: ↓ NAA/Cr FWM: No sig. diff. NAA/Cr or Cho/Cr PWM: No sig. diff. NAA/Cr or Cho/Cr No correlation with symptom severity.	Homogenous population. No partial volume correction. tCr used as the internal ref. Controlled for alcohol and medications.
8 (Kimbrell, Leulf et al. 2005)	Medial temporal lobes / 20 x 20 x 30 mm^3	1.5T/ STEAM / 2/ 30/ 128 / mixing time 13.7ms)	47 PTSD. 21 Veteran controls (no previous combat exposure).	No head injury with LOC Left handed No prior neurological illness Controls screened for psychological illness using SCID.	War related trauma Subdivided into combat and non-combat groups.	SCID (DSM-IV) CAPS WSM III WASI MAST DAST BDI SCL-90R	NG	Left MTL: NAA/Cr higher in PTSD-C vs PTSD-NC. Right MTL: no difference. No difference between NAA/Cr in the MTL between HC and PTSD. No correlation with NAA/Cr in either MTL and severity of PTSD.	Did not consider other traumas. Cr reference. No partial volume correction. Did not consider childhood abuse. Significant difference between the age of subjects and controls (44.5 vs 48.4). Controlled for handedness and prior mTBI. Matched for depression.
9 (Mahmutyazicioglu, Konuk et al. 2005)	Hippocampi / 30 x 11 x 12 mm^3 dACC / 30 x 20 x 10 mm^3 or 30 x 15 x 16 mm^3	1.5T / PRESS / 2 / 136 / 128	10 PTSD 6 Healthy controls	Alcohol or substance abuse during the last 6 months. Use of drugs of any kind Claustrophobia Pregnancy MR incompatible metal prosthesis / implant.	Antiterrorist combat Witnessing the death of a friend in a fire Childhood sexual abuse Trapped in a landslide.	CAPS	64	Hippocampi: ↓ NAA/Cr and ↑ Cho /Cr dACC: ↓ NAA/Cr and no difference in Cho /Cr.	Visual scoring (Scheltens et al) for hippocampal atrophy. No atrophy reported. Cr reference. Peaks calculated on the Phillips scanner. No partial volume correction. Small groups.

10 (Seedat, Videen et al. 2005)	ACC / 10 x 10 x 30 mm^3 Left occipital gray matter / 10 x 10 x 30 mm^3.	1.5T / PRESS / 1.5 / 135 / 256	7 female PTSD. 9 female trauma exposed controls. 11 non-trauma exposed controls.	Psychotic disorder Bipolar disorder Psychotropic medications within 6 weeks Substance use disorder within 1 year > 2 years of alcohol abuse Neurologic disorder or head injury associated with cognitive dysfunction History of seizure disorder ADD or learning disability Pregnancy or HIV.	Intimate partner violence - out of relationship for 4 months but not >2yrs.	SCID-P (DSM-IV) CTS -2 Stroop	CAPS not recorded.	ACC: ↑ Cho /Cr and ↑ mI/Cr in PTSD subjects when compared to TEC. ACC: no significant difference when PTSD/TEC's were compared to HC. Occipital GM: No difference in metabolites. No correlation with Stroop test or the severity of IPV.	tCr used as the internal ref.
11 (Li, Chen et al. 2006)	Hippocampi / 15.3 x 20.3 x 40 mm^3	1.5T / PRESS / 1 / 144 / 248	Prospective case-control study. 12 PTSD. Recent Diagnosis. 12 Trauma exposed controls.	No alcohol or other substance use disorder within 1 yr. No psychotropic medications.	Fire in Hunan Province China. All participants exposed to the trauma.	Psychiatrist interview. Diagnosed according to SCID DSM-IV. DEQ	CAPS not recorded.	Left hippocampus: ↓ NAA/Cr. Using VBM identified reduced gray matter density of the left hippocampus.	Didn't measure handedness. tCr and Cho metabolite ratios calculated. Small number of participants with major depression. No medications.
12 (Freeman, Kimbrell et al. 2006)	Hippocampi / 10 x 10 x 40 mm^3	1.5T / PRESS / 2 / 144 / 128	POW + PTSD POW – PTSD Control	Female sex Left handed History of TBI with LOC No history of neurological impairment or degenerative neurological illness Current or lifetime alcohol dependence MMSE >26.	Prisoners of war	CAPS-25 SCID Edinburgh handedness inventory Beck depression inventory Davidson trauma scale Combat exposure scale Rey Auditory Verbal Learning Test, Logical Memory Recognition Memory Test for Faces.	POW + PTSD: 53 POW – PTSD: 14 Control: 4	Hippocampi: No statistical difference in NAA/Cr or Cho/Cr in either group. No difference in hippocampal volumes.	Controlled for gender, age and education. Processed using SPARC workstation. Hippocampal volume manual determined.
13 (Ham, Chey et al. 2007)	ACC / 15 x 15 x 15 mm^3 Hippocampi / 15 x 15 x15 mm^3	3.0T / PRESS / 2 / 35 / 128	26 PTSD subjects. 25 age and gender matched HC.	Current or past significant medical illness Physical injury from fire >10% BSA burn LOC during escape Any axis I psychiatric diagnosis Antisocial or borderline personality disorder Lifetime exposure to any substance other than nicotine, moderate alcohol use and caffeine. ADHD IQ < 80 Contraindication to MRI scanning.	Fire in subway train Taegu, South Korea. February 2003.	SCID (DSM IV) CAPS Hamilton Depression rating scale Hamilton anxiety rating scale.	71	Hippocampi: ↓ NAA ACC: ↓ NAA. No difference in Cr, Cho or mI. NAA in the ACC negatively correlated with re-experiencing symptoms. Did not quantify hippocampal volume.	Absolute quantification performed with unsuppressed H_20.

14 (Schuff, Neylan et al. 2008)	Hippocampi / 7.8 x 7.8 x 15 mm^3 Frontal and parietal lobes / 8.5 x 8.5 x 15 mm^3 (two slices)	1.5T / PRESS HMRSI / 1.8/ 35 / NG	PTSD with and without alcohol abuse (28 /27). Trauma exposed controls with and without alcohol abuse (23 /26).	Past PTSD Psychotic disorder Bipolar disorder Drug abuse or dependence (last 6 months). Neurological illness Head trauma with LOC Medical disorder affecting brain function MRI exclusion criteria Brain tumour, small vessel disease, WM lesions on MRI.	Combat Military Service Civilian events Childhood abuse	CAPS SCID (DSM IV) LSC-R Total cumulative alcohol over 5 years.	CAPS +A group: 66 CAPS -A group: 63	Hippocampus: ↓NAA/Cr without smaller hippocampal volume (either group.). No significant reduction in absolute NAA. ACC: ↓NAA (absolute) Alcohol had no significant effect on the brain volumes. Trend toward smaller hippocampal volume in subjects with prior childhood trauma. Depression has no effect on the regression model.	Large groups. Noting that data for 19 subjects was not of good quality. A large number of participants in both groups had previous childhood abuse. Absolute (arbitrary units) concentration determined according to (Schuff, Neylan et al. 2001). Metabolite ratios also calculated, normalised to Cr. ACC region not further specified.
15 (Guo, Chen et al. 2012)	Hippocampi / 10 x 10 x 10 mm^3 ACC / 10 x 10 x 10 mm^3	1.5T / PRESS / 1 / 144 / NG	50 PTSD. 50 heathy controls. Age and gender matched groups.	Antipsychotics, antimanic, antidepressant or benzodiazepine drugs. History of LOC > 5 minutes Clear diagnosis of neurological disease Serious body disease Clear diagnosis other mental disorder History of alcohol or morphine abuse History of childhood abuse.	PCL score >= 44 CAPS > 60 <1-year duration of PTSD	PCL-C CAPS WAIS	CAP not reported	ACC: ↓NAA/Cr and ↓Cho/Cr. Hippocampi: ↓NAA/Cr.	ACC location not further specified. Data analysis performed using Functool 2 and ACD 4.0. tCr used as the internal ref. Volume quantification not performed.
16 (Eckart, Kaufmann et al. 2012)	Hippocampi (medial and posterior portion) / 20 x 10 x 10 mm^3 Bilateral insula / 30 x 10 x 15 mm^3	3.0T / PRESS / 2 / 135 / 256	20 PTSD. 16 Trauma exposed controls. 11 Healthy controls. All male.	Lifetime or current abuse of substances (particularly alcohol) Neurologic diseases Any contraindication for magnetic resonance imaging (MRI) Psychiatric conditions other than PTSD or major depression.	Highly traumatised refugees. Trauma exposure occurred at mean age of 15.	CTQ Vivo checklist of War, Detention and Torture Events CAPS MINI MP test	CAPS PTSD: 69 CAPS: TEC: 14	No significant difference in the absolute NAA or NAA/Cr between PTSD and HC in any brain region. No significant difference in the absolute NAA or NAA/Cr between PTSD and TEC in any brain region. No difference in the hippocampi or the insula.	Absolute quantification performed with unsuppressed H_20. LC Model. Partial volume correction performed. Volume quantification of hippocampi and insula. Only one PTSD subject was taking psychoactive medications. Co-morbid major depression (n=15 PTSD). Inclusion of depression as a co-variate did not alter results.
17 (Shu, Xue et al. 2013)	Hippocampi / 7.5 x 7.5 x 10 mm^3	1.5T / PRESS-CSI / 1/ 144 / 2	11 PTSD (right handed). 11 age, gender and education matched HC.	Bipolar disorder Schizophrenia or psychotic disorder Alcohol or Substance abuse or dependence in past 5years Physical or sexual abuse during childhood	Mixed: including sexual assault.	CAPS	Mean CAPS 84.9	Hippocampi: ↓ NAA/Cr No difference in Cho. Significant reduction in left and total normalised hippocampal volume when compared to controls.	PTSD group receiving anti-depressants and anti-psychotics. tCr used as the internal ref.

				Current or past history of neurological disease Other physical diseases identified by EEG or MRI.				Volume of the left hippocampus was negatively correlated with CAPS total and CPAS-C scores. Serum cortisol negatively correlated with right hippocampal NAA/Cr ratio.	GE ADW Functool used to quantify peaks area. Controlled for handedness, age, gender and education.
18 (Rosso, Weiner et al. 2014)	Right Insula / 15 x 30 x 20 mm^3 dACC / 30 x 20 x 20 mm^3	4.0T / MEGA-PRESS / 2 / 68 / 384	13 PTSD. 13 Healthy controls.	Medical condition: mTBI, LOC, seizures Current substance use disorder Nicotine use Use of benzodiazepines or anxiolytic anticonvulsant Mood stabiliser or neuroleptic medication within 4 weeks of the study Substance abuse in the last five years Structural abnormality on MRI scan Recent psychoactive drug (urine screen). Pregnancy.	Assault Childhood physical abuse Childhood sexual abuse Combat exposure Rape Multiple traumas	SCID - I CAPS STAI-S/T BDI	62	Insula: ↓ GABA/Cr. No association with symptom severity. Negative correlation with state and trait anxiety. dACC: GABA/Cr: no difference between groups. No difference in Cr or NAA.	Voxel located within the dACC. Spectrum pre-processed and then fitted using LC Model. tCr used as the internal ref. Matched for menstrual cycle. Almost all patients free of psychotropic medications. Controlled for drugs, nicotine and alcohol.
19 (Pennington, Abe et al. 2014)	rACC / 35 x 25 x 20 mm^3 POC / 40 x 20 x 20 mm^3 TEMP / 20 x 40 x 20 mm^3	4.0T / MEGA-PRESS / 2 / 68 / 384 4.0T / STEAM / NG / 16 / NG	10 PTSD with AUD. 28 PTSD without AUD. 20 Trauma exposed controls.	Schizophrenia or schizoaffective disorder Past or current alcohol dependence (control) Suicidal intention Bipolar Suicidal ideation Seizure disorder Head injury with (post injury mem loss >24hrs or LOC 10min) Stroke Neurodegenerative condition HIV Medically unstable Psychotropic medication last two weeks.		CAPS ISI AUDIT Time line follow back Beck Depression Inventory - II Beck anxiety inventory.	PAUD: 78.6 PTSD: 55.2 CON: 2.7	TEMP: lower NAA in PAUD than PTSD and TEC. No diff PTSD vs TEC. TEMP and ACC: ↓ Cho in PAUD compared to TEC and PTSD. rACC: ↓ mI in PAUD in ACC when compared to TEC and PTSD. TEMP: ↑ Glx in PAUD vs PTSD. No difference PAUD vs. TEC. POC: ↑ GABA in PAUD vs PTSD. No difference PAUD vs. TEC. Within PAUD, higher GABA and Glu correlated with improved cognition.	Absolute concentrations (IU). ACC voxel contained primarily within the rostral ACC.
20 (Michels, Schulte-Vels et al. 2014)	DLPFC / 25 x 40 x 30 mm^3 rACC / 28 x 30 x 22.5 mm^3	3.0T / MEGA-PRESS / 1.8 / 68 / (320 DLPFC and 640 ACC)	12 PTSD (Some on medication). 17 Trauma exposed controls.	Free of neurological and other major medical conditions.	Mixed	Trauma History - PDS Childhood trauma questionnaire SCID-I SCID-I-R CAPS Multidimensional inventory of dissociation State trait anxiety inventory Beck depression inventory Cognitive testing	67	DLPFC and rACC: ↑ GABA/Cr and ↑ glutathione/Cr. No difference in NAA/Cr or Glx/Cr. Positive correlation with glutathione and disease duration.	Voxel located primarily within the rostral ACC. tCr used as the internal ref. Fitted using LC model.

21 (Meyerhoff, Mon et al. 2014)	rACC / 35 x 25 x 20 mm^3 POC / 40 x 20 x 20 mm^3 TEMP / 40 x 40 x 20 mm^3	4.0T / STEAM / 1.8 / 15 / 128 4.0T / MEGA-PRESS / 2 / 71 / 256	Adults - Military and civilian population. 27 PTSD. 18 Trauma exposed controls.	Schizophrenia or schizoaffective disorder Alcohol dependence within 6 months Bipolar Suicidal ideation Seizure disorder Head injury with (post injury symptoms or LOC 10min) Stroke Neurodegenerative condition Medically unstable injuries related to trauma.	Combat (n=17) Non-combat (n=10)	CAPS PCL Insomnia severity index (ISI) AUDIT FTND BDI BAI STAI SCL-GSI	PTSD: 54.5	rACC: ↓NAA, no difference in Glu and GABA. TEMP: ↓GABA and ↑ Glu. POC: ↓ GABA and NAA and Glu no difference. No difference in Cr, Cho or mI in any of the groups. In PTSD POC GABA was negatively correlated with ISI (r= -0.55, p=0.008).	Absolute concentration (IU). Quantified with 'SI tools' Changed the number of averages depending in SNR (192 vs 256)
22 (Yang, Quan et al. 2015)	rACC / 20 x 20 x 20 mm^3	3.0T / PRESS / 2 / 30 / 128	Adolescents. 23 Remitted PTSD. 10 Healthy controls. 10 PTSD.	Smoking Alcohol Excess Other axis I disorder (except mood / anxiety) IQ<80 Psychotropic medication last 4 weeks Any sig. medical or neurologic condition.	Earthquake	IQ - Wechsler Intelligence Scale for Chinese Children CAPS-CA C-WISC	PTSD: 68.7 Remitted: 5.6	rACC: Lower Glx/Cr between PTSD group and HC. Glx lowest in PTSD followed by patients in remission. No difference in NAA/Cr, Cho/Cr, Glx/Cr was weakly correlated (r=-0.355, p=0.314) with CAPS-CA.	Peaks normalised to Cr. Fitted using AMARES.
23 (Harnett, Wood et al. 2017)	dACC / 20 x 27 x 10 mm^3	3T / PRESS / 2 / 30 / 128	19 Trauma exposed individuals (physical injury requiring visit to trauma unit / emergency and exposure to traumatic event on the PDS) 18 Healthy controls	History of blood or circulation disorders Diabetes Brain or spinal abnormalities Pregnancy Previous or current head injury Previous / current diagnosis of mental illness prior to event.	Physical injury requiring visit to trauma unit / emergency and exposure to traumatic event on the PDS.	PDS (at T_0 and T_1) PRFS WTAR	PDS (1 month): 13 PDS (3 month): 12.15	No difference between the groups. Glx Positive linear relationship and current stress disorder symptoms in trauma exposed participants. Glx positive linear relationship with future stress disorder symptoms.	Significant difference in participant age (4.5 years) Absolute concentration. Metabolites normalised to water. Partial volume correction performed.
24 (Rosso, Crowley et al. 2017)	Hippocampi / 15 x 20 x 30 mm^3	4.0T / 2D JPRESS / 2 / 30 – 330ms / 16 (4 scans per TE)	24 PTSD. 23 Trauma exposed controls. Age and gender matched. Female participants matched for menstrual cycle.	Medical conditions affecting brain structure Current substance use disorder Current nicotine dependence Anxiolytic, anticonvulsant, mood stabilizing or neuroleptic medication use within 4 weeks of study History of substance abuse within the past 5 years Lifetime history of substance dependence Lifetime history of psychosis MR contraindications Urine test positive for psychoactive drugs or beta-HCG.	Childhood abuse (n= 5) Childhood sexual abuse (n=4) MVA or violent accident (n=2) Victim of physical assault (n =7) Combat exposure (n=1) Victims of sexual assault (n=9).	SCID / IP CAPS Traumatic life events questionnaire.	PTSD: 59.5	Hippocampi: ↓ NAA/H_20 Right hippocampus: ↑ Glu/H_20 and Glu/NAA Re-experiencing symptoms were negatively correlated with all NAA ratios. Significant positive correlation between re-experiencing symptoms and the right hippocampus Glu / H2O not for Glu/Cr. Trauma load was significantly positively correlated with right Glu /NAA in PTSD. No significant difference in the gray matter volume.	FWHM 8-12 Hz Metabolites were normalised to both water, Cr and NAA.

Table 5 - A summary of the methods and results of the MRS studies included in this review. Abbreviations: 2D-JPRESS – two-dimensional J-resolved spectroscopy; ADHD – attention deficit hyperactivity disorder; AUD: Alcohol use disorder; beta-HCG – Human chorionic gonadotropin (pregnancy marker); BSA – body surface area; DLPFC – Dorsolateral prefrontal cortex; EEG – electroencephalogram; FWM – Frontal white matter; HC – healthy control; IQ – intelligence quotient; K-SADS-PL - Schedule for Affective Disorders and Schizophrenia for School-Age Children-Present and Lifetime version; LOC – loss of consciousness; mTBI – mild traumatic brain injury; NA – not applicable; NG – not given; PAUD – PTSD with alcohol use disorder; PCL – PTSD check list; PDS – post traumatic diagnostic scale; POC – Posterior occipital cortex; POW – Prisoner of war; PRESS – Point-RESolved Spectroscopy; PRFS – Psychosocial risk factor survey; PWM – Parietal white matter; STEAM – Stimulated Echo Acquisition Mode; TEC – trauma exposed control; TEMP – lateral temporal cortex; WM – white matter; WTAR – Weschler test of adult reading;. Please go to the individual manuscripts for further details of psychological assessment.

Neurochemical Deregulation in Posttraumatic Stress Disorder identified using 1D and 2D-LCOSY spectroscopy

Scott Quadrelli, Nathan Tosh, Aaron Urquhart, Katie Trickey, Rosanna Trewan, Graham Galloway, Lisa Rich, Rodney Lea, Peter Malycha, Carolyn Mountford.

Abstract

Posttraumatic stress disorder (PTSD) is triggered by experiencing terrifying event(s) for which there is currently no objective test for a definitive diagnosis. We report a preliminary study where one-dimensional (1D) and two-dimensional (2D) neuro magnetic resonance spectroscopy (MRS), collected at 3T in a clinical scanner with a 64-channel head coil, identifies neuro deregulation in the PTSD cohort. The control subjects (n=10) were compared with PTSD participants with minimal co-morbidities (n=10). Using 1D MRS we identified reduced inositol in the PCC, which was positively correlated with hyperarousal symptoms. Additionally, 1D MRS identified increased glutamate in the ACC raising the possibility that increased glutamate may result in excitotoxicity in this region. The 2D L-COSY identified statistically significant increases in the total spectral region containing both free substrate fucose and fucosylated glycans of 31% ($p = 0.0013$), two of multiple fucosylated glycans (Fuc IV and VI) were elevated by 48 % ($p = 0.002$) and 41 % ($p = 0.02$) respectively, imidazole was increased by 12 % ($p = 0.002$), and lipid saturation was increased by 12.5% ($p = 0.009$). This is the first evidence of fucosylated glycans, reported in animals to be involved in learning and memory, to be affected in humans with PTSD.

Introduction

Posttraumatic stress disorder (PTSD) is a mental health condition triggered by either experiencing or witnessing a terrifying event(s). Symptoms may include flashbacks, nightmares and severe anxiety, as well as intrusive thoughts about the trauma. A large percentage of the population will witness traumatic events during their lives, and most do not develop PTSD. However, for approximately 5-30% of trauma exposed individuals PTSD develops (Breslau, Kessler et al. 1998, Davidson, Stein et al. 2004), characterised by persistent re-experiencing of the traumatic event, avoidance of stimuli related to the

trauma, and hyperarousal for at least one month (American Psychiatric Association 1994), leading to significant psychosocial impairment for patients. PTSD is associated with multiple confounding organic and psychiatric conditions, such as depression, anxiety, cardiovascular disease and chronic pain (Brady, Killeen et al. 2000, Boscarino 2012, Siqveland, Ruud et al. 2017).

1D spectroscopy has previously been applied to PTSD in an effort to quantify metabolic abnormalities in the brains of patients. The most consistent abnormality identified in PTSD is reduced NAA/Cr, in the anterior cingulate cortex (ACC), hippocampus / medial temporal lobe, posterior occipital cortex (POC), and several other brain regions (see Systematic Review Chapter – 4). Customised 1D spectroscopy sequences, such as MEGA-PRESS (Mescher, Merkle et al. 1998) have also been used to measure glutamine, glutamate and GABA in various brain regions (Michels, Schulte-Vels et al. 2014). Multiple studies have now identified evidence of glutamatergic dysfunction and deregulation of GABA in some of the brain regions, that based on previous fMRI work, we would expect hypo-activation, such as the occipital cortex and ACC (Michels, Schulte-Vels et al. 2014, Pennington, Abe et al. 2014, Yang, Quan et al. 2015). However, 1D spectroscopy has a number of limitations, such as the number of metabolites that can be quantified, due to overlapping resonances from metabolites such as glutamine, glutamate and GABA.

Two-dimensional (2D) MRS is a technique that allows composite or overlapping resonances from 1D spectra to be separated out. In conventional 1D spectroscopy, intensity (y-axis) is plotted against frequency (x-axis), whereas in 2D spectroscopy intensity is plotted against two frequency variables (Keeler 2010). The introduction of 2D *in vivo* spectroscopy has allowed researchers to make unambiguous metabolite assignments, that could not have been made using 1D spectroscopy (Thomas, Yue et al. 2001, Ramadan, Ratai et al. 2010, Ramadan, Andronesi et al. 2011, Mountford, Quadrelli et al. 2015). 2D Localised COrrelation SpectroscopY (L-COSY) has been shown to be a reliable method for *in-vivo* detection of brain metabolites (Binesh, Yue et al. 2002, Arm, Al-iedani et al. 2018). New neurochemical assignments have been made possible using the 2D COSY protocol with the latest hardware capabilities. These include multiple fucose-α(1–2)-glycans and the substrate α-fucose in the spectral region F2, 3.95–4.50 ppm; F1, 0.90– 1.70ppm (Mountford, Quadrelli et al. 2015). From animal studies, a growing body of literature implicates fucose-α(1–2)-glycans in the molecular mechanisms that underlie neuronal development, learning and memory in the brain (see chapter 2)

We acquired 2D L-COSY data from the posterior cingulate cortex (PCC), part of the posteromedial cortex, a highly connected and metabolically active brain region (Leech and Sharp 2014). This region

has been previously implicated in conditions such as Alzheimer's and traumatic brain injury (TBI) (Greicius, Srivastava et al. 2004, Bonnelle, Leech et al. 2011). The PCC is hypothesised to provide a link between the hippocampal formation and the higher-level cortices and may play a role in the balance between internal and external thought (Leech, Kamourieh et al. 2011). Additionally, the PCC responds to emotional stimuli and may integrate this with higher order processing (Maddock, Garrett et al. 2003, Leech and Sharp 2014).

We hypothesized that 2D spectroscopy would provide additional metabolic information, not available using traditional 1D MRS. We also aimed to determine if there were unique metabolite alterations in the PCC, a highly connected component of the limbic network, in patients with PTSD when compared to healthy controls.

Materials and Methods

Participants

As part of this case control study, we recruited 20 participants, 10 adults with PTSD and 10 healthy controls, from a number of sources including newspaper advertisement, local psychiatrists and psychologists. PTSD subjects were eligible if they had been diagnosed with PTSD according to the DSM-IV using the Clinician- Administered PTSD scale (CAPS) (Blake, Weathers et al. 1995) and were aged between 18-65 yrs.

Exclusion criteria included: current substance use disorder; lifetime history of substance use disorder; current or past history of schizophrenia, bipolar or other psychotic disorder; major head injury; current or past history of neurological disease; current pregnancy or contraindication to MRI scanning. Healthy control participants were included if they were aged between 18-65 years and had no current DSM-IV Axis I disorder, as assessed by the Structured Clinical Interview for DSM IV (SCID) (First 2002) and no lifetime history of a mood or anxiety disorder, major head injury; current or past history of neurological disease; current pregnancy or contraindication to MRI scanning. The groups were matched for age, gender and education as shown below in Table 6.

Written informed consent was obtained from all participants prior to study commencement and the research protocol was approved by the Hunter New England Local Health District, Metro South Health and Metro North Health human research ethics committees.

Self-Report Measures and Clinical Interviews

Prior to MRI scanning participants completed an online questionnaire that contained: Ohio State TBI Identification questionnaire (Corrigan and Bogner 2007); Alcohol Use Disorders Identification Test (AUDIT) (Babor, De la Fuente et al. 1992); Generalised Anxiety Disorder 7-item (GAD-7) scale (Spitzer, Kroenke et al. 2006); Primary Health Questionnaire -15 (PHQ-15) (Kroenke, Spitzer et al. 2002); Primary Health Questionnaire – 9 (PHQ-9) (Kurt Kroenke 2002); Kessler Psychological Distress Scale (K10) (Kessler, Andrews et al. 2002); PTSD checklist (PCL-C) (Weathers, Litz et al. 1993); and Chronic Pain Intensity Scale (Von Korff, Ormel et al. 1992), the questionnaire can be found in Appendix 1.

A clinical psychologist interviewed all participants and administered the CAPS, SCID/IP, Life Events Checklist (LEC) (Weathers 2013) and Ohio State TBI Identification questionnaire. PTSD participants reported the following traumas: occupational traumatic exposure – emergency services (*n* =2); occupational traumatic exposure – police officer (*n* = 5); remaining (*n* = 3). Nine PTSD participants had current major depressive disorder (MDD) and one had past MDD. Two PTSD participants were unmedicated, the medications being taken by the remaining participants are detailed in Table 6.

MRI Methods

All scans were performed on a 3T Prisma scanner (Siemens, Erlangen, Germany, software version VD13D) with a 64-channel head and neck coil (Siemens, Erlangen) at one of two sites: Hunter Medical Research Institute (NSW, Australia) and Hurston Imaging Research Facility (QLD, Australia).

Structural Imaging

A three-plane localizer image was performed for volume of interest placement. After global shimming, a 3D T1-weighted magnetization-prepared rapid gradient-echo (MPRAGE) was acquired (TR/TE/TI=2530/3.5/1100ms, flip angle=7°, field of view=256x256 mm, voxel size 1x1x1mm3, IPAT=3, acquisition time 4:28 minutes) that was used for MRS voxel placement and whole brain morphometry. The T1 3D-MPRAGE was reconstructed in sagittal and coronal planes with 1mm slice resolution for accurate localization of MRS voxels.

Localised COSY 2D MR Spectroscopy

L-COSY data were acquired from a 3x3x3 cm^3 voxel positioned in the posterior cingulate cortex (PCC) as shown below in Figure 9. The PCC is made up of WM and GM and was chosen partly due to the role it plays in learning and memory (Mountford *et al.*, 2015) and due to the favourable magnetic field shimming and its relative insensitivity to motion during spectral acquisition.

L-COSY was acquired with the following parameters: RF carrier frequency at 2.0 ppm; TR 1.5 s; water suppression using WET; 96 t1 increments; with 8 averages per increment, acquired vector size 1024 points; acquisition time 512 ms; spectral width in F2 2000 Hz and spectral width in F1 1250 Hz (0.8 ms increment size). Time of acquisition was 19 minutes.

Localised shimming was undertaken by adjustment of zero- and first-order shim gradients using the automatic B0 field mapping technique supplied by the vendor (Siemens AG) followed by manual adjustment of accessible shim gradients to achieve a resulting peak width of water at half-maximum that was 15Hz or less.

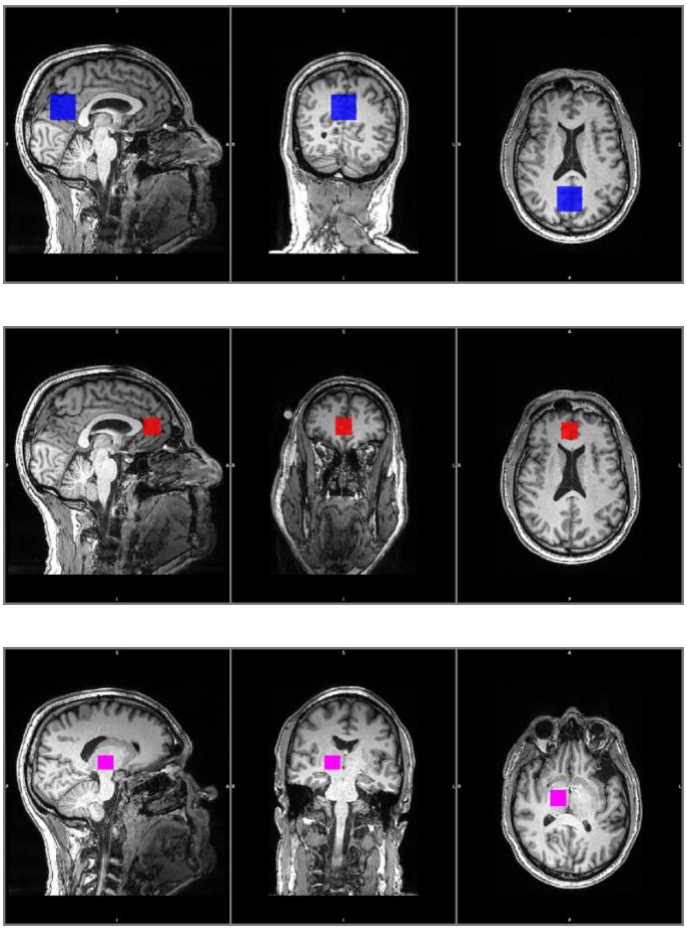


Figure 9 – Representative voxel location in the PCC (top), ACC (middle) and right thalamus (bottom).

1D Spectroscopy

1D proton spectra were acquired from three separate brain regions: anterior cingulate gyrus (ACC); right thalamus and PCC. The ACC VOI was located in the midline, just anterior to the genu of the corpus callosum and measured 20 x 20 x 20 mm3. The thalamus VOI was centred within the thalamus after reviewing all three imaging planes and measured 1.9 x 1.9 x 1.7 cm3. The PCC VOI was the same for both 1D and 2D and is described above. Spectral data were acquired using Point-Resolved Spectroscopy (PRES), acquired with the following parameters: TR/TE: 1500/30ms, 96 averages, bandwidth 1200 Hz, delta frequency -2.3ppm, 1024 points. Acquisition time was 3:22. Shimming was performed prior to each acquisition as described above for L-COSY, to ensure a linewidth less than 15 Hz.

Spectral Quantification

All participants had satisfactory L-COSY data for analysis. Raw L-COSY data were transferred to MATLAB (MathWorks 1984-2014) for signal combination from multiple elements followed by row concatenation into a 2D matrix (MathWorks 1984-2014) for signal combination from multiple elements followed by row concatenation into a 2D matrix. Commercial 2D spectral processing software (Accelrys Felix NMR, 2007) was used for observer-independent spectral processing and analysis. The processing parameters used were: F2 domain (skewed sine-squared window, 2048 points, magnitude mode), F1 domain (sine-squared window, linear prediction to 96 points, zero-filling to 512 points, magnitude mode). No additional water removal was applied in Felix since water was sufficiently suppressed during acquisition. The total creatine methyl diagonal resonance at 3.02 ppm was used as an internal chemical shift reference in F1 and F2, absolute quantification of Cr was performed using 1D MRS in LCModel to ensure Cr was unchanged between the two groups.

1D MRS datum was fitted using LCModel (Provencher 1993) – version 6.2-2B, based on a vendor provided simulated basis set obtained using a GAMMA library. Eddy current correction was performed within LCModel by using unsuppressed water signal acquired from same location. Metabolites with a Cramer-Rao lower bounds (CRLB) of >20% were not included in the final analysis. Next, tissue-type segmentation with partial volume estimation is carried out (Zhang, Brady et al. 2001) in order to calculate total volume of brain tissue, including separate estimates of volumes of grey matter, white matter, peripheral grey matter and ventricular CSF, as described in Chapter 3.

Statistical Analysis

Metabolite means for each test group were compared using the Mann-Whitney tests, since the distribution of some metabolites exhibit partial deviation from normality. Correlation analyses between metabolites and clinical variables was performed using Spearman's correlation.

Results

Demographics

There was no significant difference between the mean group age, gender, or years of education as shown below in Table 6.

Table 6 - Demographic and clinical characteristics of the PTSD and healthy control participants

Characteristic	PTSD (n = 10)	Control (n=10)
Age (years)	44.7 ± 11.36	44.4 ± 11.08
Education (years)	14.4 ± 2.4	15 ± 2.9
Sex (Female)	5 (50)	5 (50)
CAPS – total	38.4 ± 10.45	
CAPS - intrusion	8.4 ± 0.95	
CAPS – hyperarousal	11.8 ± 1.11	
CAPS - avoidance	5.4 ± 0.48	
Medications		
Clonidine	2	
Prazosin	2	
Propranolol	1	
Sertraline	1	
Desvenlafaxine	1	
Escitalopram	1	
Amitriptyline	2	
Venlafaxine	1	
Abbreviations: TLEC – Traumatic life events checklist; CAPS; AUDIT; PCL ; K10; PHQ -15; PHQ – 9; GAD -7. **Mean ± SD or N (%)**		

2D-COSY

All participants had sufficient quality L-COSY data for analysis. A typical 2D-COSY spectrum for a healthy control volunteer and a PTSD participant is shown in Figure 10. The spectral region containing the expanded total fucose region (F2, 3.97–4.47 ppm; F1, 0.90–1.72 ppm) is shown in Figure 11. A summary of the statistically significant neurochemical differences is shown in Table 7. Statistically significant increases were recorded in IMI-1 of 12%; total Fucose region of 31 %; Fuc IV of 48 %; Fuc VI of 41 %; and an increase in the lipid cross-peak B, which is the HC=CH-CH2- CH2- CH3 of lipid fatty acyl chain, of 12.5%. No significant differences were recorded for NAA, glutamine, glutamate, myoinositol or GABA using the 2D-LCOSY method.

Table 7 - Metabolite levels (mean +/- SD) in the posterior cingulate cortex identified with 2D L-COSY.

Metabolite	PTSD	Control	Average Chemical Shift (F2-F1) ppm
IMI-1	2.66 ± 0 .22	3.21 ± 0.44 ** *MWU	7.07 – 7.07
t-Fucose	5.83 ± 1.36	7.62 ± 1.34** *MWU	3.95 – 4.50 ppm; -0.90 – 1.70 ppm
Fucose IV	0.46 ± 0.12	0.69 ± 0.18** *MWU	4.36 – 1.35
Fucose VI	0.43 ± 0.09	0.61 ± 0.19* *MWU	4.45 – 1.35
Lipid HC=CH-CH2- CH2- CH3	5.09 ± 0.55	6.38 ± 1.26** *MWU	1.37 – 1.99

(IMI-1) - imidazole from histamine, histidine, and homocarnosine.

Significance shown is derived using a 2T Students T tests with * P<0.05, **P<0.01, ***P<0.001.

- ***MWU Indicates significance (P<0.05) using Mann Whitney U tests.**

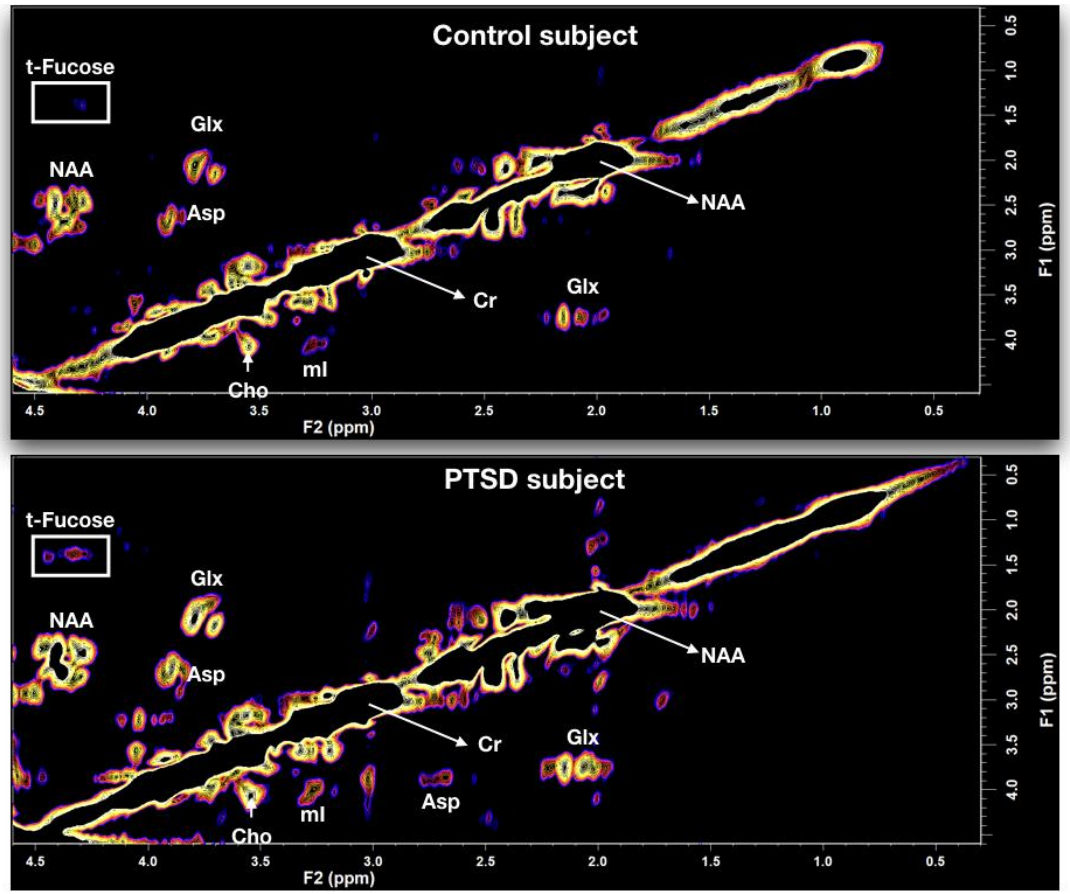


Figure 10 - In vivo localised correlated spectroscopy (L-COSY) of the human brain (posterior cingulate cortex) acquired at 3 T using a 64-channel head coil; voxel size, 30 × 30 × 30mm3. Asp, aspartate; Cho, choline; Cr, creatine; Glx, glutamate and glutamine together; m-Ino, myo-inositol; NAA, N-acetylaspartate. The region highlighted by the white box is expanded in Figure 11. Upper: healthy control and Lower: patient with PTSD.

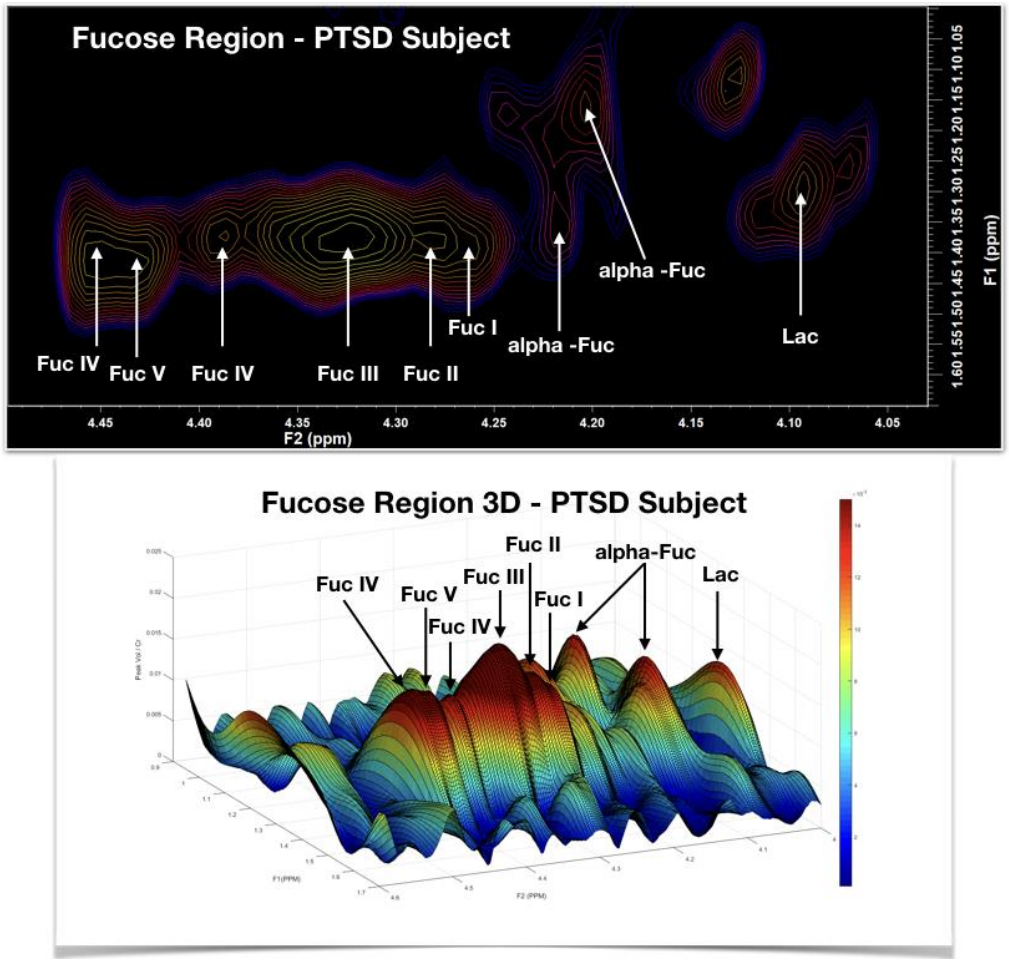


Figure 11 – Expanded Fucose region (F2, 4.03–4.5 ppm; F1, 1.05–1.65 ppm) of the localised correlated spectroscopy (L-COSY) in Figure 10 with assignments of fucose I (Fuc I) to fucose VI (Fuc VI) denoted. Upper: Contour plot produced using Felix software. Lower: Three-dimensional plot of the same dataset produced using MATLAB.

1D MRS

LC-model was able to fit 1D MRS data for up to 35 lipids, metabolites and macromolecules and example spectrum from PTSD and healthy control subjects are shown below in Figure 12. There were no significant group differences between absolute tCr for all brain regions. The significant metabolite ratio group differences identified using 1D spectroscopy are tabulated below in Table 8, for each brain region.

Inositiol was found to be significantly reduced in the PCC when referencing to H2O, total creatine and water. Glutamate/tCr and Glx/tCr was significantly increased in the ACC. No statistically significant mean differences were identified between the groups from metabolites measured in the right thalamus.

Table 8 - Metabolite levels measured using 1D MRS in the ACC and PCC in PTSD and HC Participants.

Voxel	Metabolite	PTSD	Control	p-value *
PCC	Inositol/H_2O*	4.08 ± 0.47	4.68 ± 0.42	0.007
PCC	Inositol/tCr	0.8 ± 0.08	0.9 ± 0.08	0.015
PCC	Inositol/NAA	0.55 ± 0.06	0.63 ± 0.05	0.009
ACC	Glu/tCr	1.51 ± 0.14	1.33 ± 0.15	0.011
	Glx/tCr	1.67 ± 0.11	$1.49 \pm .2$	0.015

*** Indicates significance using Mann Whitney U tests.**

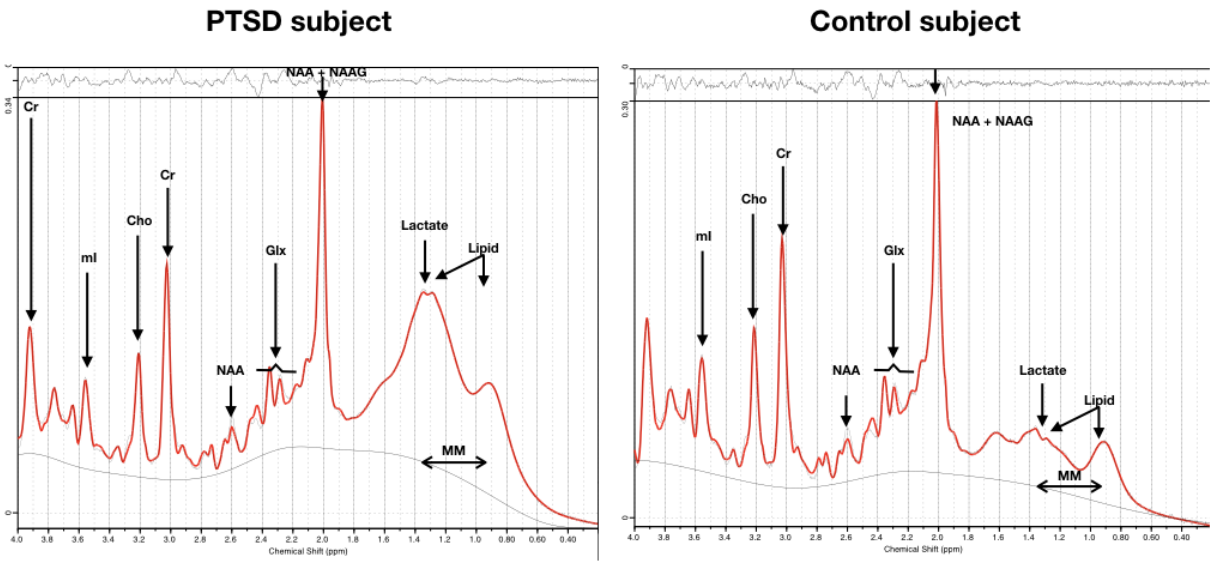


Figure 12 - 1D spectrum from a PTSD (left) and healthy (right) control subject acquired from the PCC.

Correlation of metabolites with clinical measures

Levels of IMI-1 a metabolite identified using 2D L-COSY were significantly positively correlated with hyperarousal symptoms (r=0.66, p=0.04) (Figure 13). Levels of inositol in the PCC, Glu in the ACC and Glx in the ACC and were not correlated with disease severity measures.

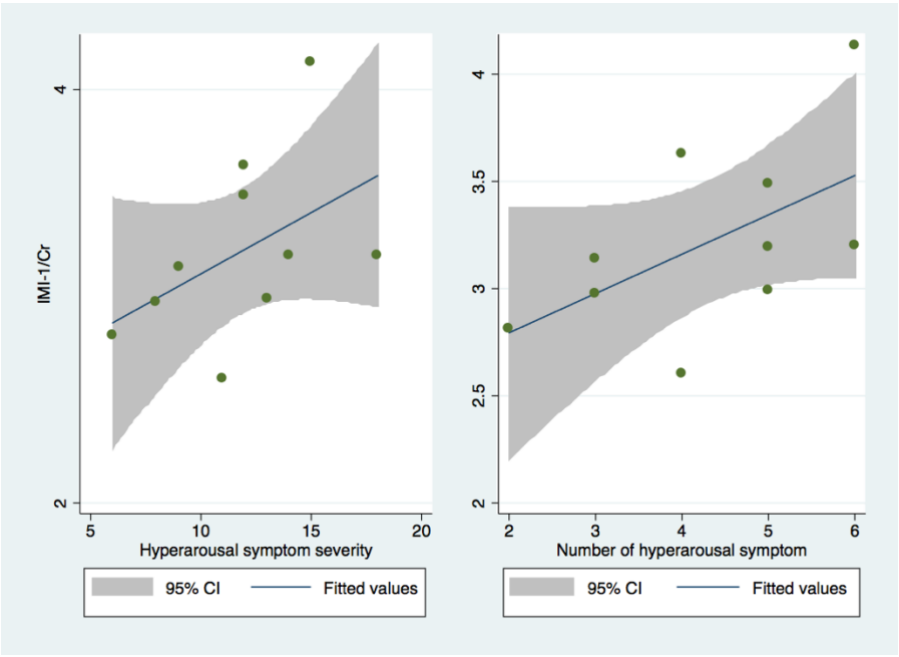


Figure 13 - Correlation between hyperarousal symptoms of PTSD in PCC and IMI-1/Cr.

Discussion

We applied a novel *in-vivo* spectroscopy technique, 2D-L-COSY to a region of the brain that to our knowledge has not previously been interrogated in PTSD. We aimed to enhance the chemical information available using MRS in PTSD. We also used single voxel 1D MRS to investigate metabolic changes in the PCC, ACC, and right thalamus.

The 2D-LCOSY identified increases in multiple neurochemicals in the PCC region of the human brain in people suffering from PTSD. The total fucose spectral region, was increased by 31%. Two of the fucose-α(1–2)-glycans assigned thus far (Mountford, Quadrelli et al. 2015), Fuc IV, was elevated by 48% and Fuc VI was elevated by 41%. In the brain fucosylated glycans are thought to be contained within synapsin proteins (Hart 2006) and regulate the release of neurotransmitters at the synapse (Evergren, Benfenati et al. 2007). Revest et al 2010 found that blocking the fucosylation of synapsin Ia/Ib inhibits the glucocorticoid mediated increase in stress related memories in the hippocampus (Revest, Kaouane et al. 2010). Suggesting that fucosylated glycans may play a role in the pathogensis of PTSD. Additionally, neuronal glycan proteins modified with fucose have shown to be important in learning and memory (Hart 2006) and are implicated in synaptic plasticity. Thus, here we provide the first *in vivo* evidence in the human brain that a specific fucose-α(1–2)-glycan is affected by PTSD and may play a role in its pathogenesis and symptoms.

The composite imidazole diagonal resonance of histamine, histidine, and homocarnosine was increased by 12% and the HC=CH-CH2- CH2- CH3 of the MR visible lipid fatty acyl chain is increased by 13%. It is not possible to determine if the recorded change in the acyl chain is due to alterations in the triglyceride, cholesterol ester or ether linked fatty acyl chain population or a mixture thereof. Lipids are normally membrane bound and are not typically visible within the MR spectrum unless there is abnormal pathology (Mountford, Stanwell et al. 2010).

We found reduced inositiol in the posterior cingulate cortex using 1D MRS. Myo-Inositol (mI) is the most abundant form of inositol found in the brain (Govindaraju, Young et al. 2000). Using 1H-MRS, mI has a prominent mutiplet peak at 3.52 and 3.61 ppm (Govindaraju, Young et al. 2000) and is thought function as an osmolyte and in the correct setting to be a astroglial marker (Brand, Richter-Landsberg et al. 1993). Only two previous studies have reported changes in mI in PTSD. Seedat et al found mI to be raised in the ACC in a cohort of participants with PTSD a result of intimate partner violence (Seedat, Videen et al. 2005). However, in a single study performed in PTSD patients with AUD, mI was found to be reduced in the ACC (Pennington, Abe et al. 2014). No significant difference in mI was identified using

L-COSY, this may relate to the reduced signal to noise of the cross peak reducing significance in this smaller cohort.

Increased Glu and Glx to creatine ratios were identified in PTSD compared to healthy controls, in the ACC using 1D MRS. Glu functions mainly as an excitatory neurotransmitter and is the most abundant amino acid in the human brain (6-13mmol $\mathrm{kg}_{\mathrm{ww}}^{-1}$) (Govindaraju, Young et al. 2000) and is thought to result in neuronal damage in excess, a process known as excitotoxicity (Olney 1986). Excitotoxicity has been implicated in neuronal cell death in multiple pathologies, including MS and epilepsy (Lewerenz and Maher 2015). Our results are supported by Pennington et al who found increased Glx in the ACC in patients with PTSD and alcohol use disorder (AUD) when compared to PTSD patients without AUD (Pennington, Abe et al. 2014). Meyerhoff et. al. also found increased Glx in the ACC and the right medial temporal cortex, with an associated increase in GABA in the same region (Meyerhoff, Mon et al. 2014), raising the possibility of glutamatergic dysfunction in these regions. More recently Rosso et al identified increased Glu concentrations in the hippocampi, associated with reduced NAA suggesting reduced neuronal integrity (Rosso, Crowley et al. 2017). It is possible that increased Glu detected using MRS in the ACC and hippocampus results in excitotoxicity, eventually manifesting as reduced parenchymal volumes (Woodward, Kaloupek et al. 2006) (Kitayama, Vaccarino et al. 2005, Smith 2005).

This study has several limitations. PTSD has multiple co-morbidities and researchers have tried recruiting participants with minimal co-morbidities, to ensure that the cohort best represents the condition of interest. However, it can be argued that this approach may not be representative of the condition itself. During recruitment, we have included participants with co-morbidities that are known to be highly associated with PSTD in an effort to be able to generalise our results to a 'like-like' PTSD population, more relevant to clinical practice. Due to the pilot nature of this study the groups are small, somewhat limiting the conclusions that can be made. However, metabolic differences between the cohorts are large, between 12 and 48 percent. There are currently several limitations of the COSY sequence, limiting its applicability to research, the main limitation is acquisition time. Multiple groups are currently trying to solve this problem using techniques such and non-uniform sampling.

Conclusion

PTSD patients with trauma resulting from occupational traumatic exposure in emergency services and the police force, were evaluated and compared with age and gender matched healthy controls using in vivo neuro 1D and 2D MR spectroscopy in a clinical 3T MR scanner. Using 1D MRS we identified reduced inositol in the PCC, which was positively correlated with hyperarousal symptoms. Additionally, 1D MRS

identified increased Glx in the ACC, which may contribute to atrophy in this region via excitotoxicity. The 2D MRS method identified multiple differences in chemical signatures in PTSD. This includes an increase in two fucose-α(1–2)-glycans as well as the appearance of the substrate α fucose. The fucose-α(1–2)-glycans have been implicated, by others using animal models, in the molecular mechanisms that underlie neuronal development, learning and memory . This is the first evidence of fucose-α(1–2)-glycan involvement in the PTSD pathogenesis in the human brain. Other differences recorded include an increase in imidazole from either histamine, histidine or homocarnosine and an increase in the level of unsaturation in a lipid fatty acyl chain.

Funding

This study was funded by the Department of Defence of Australia and Department of Defence USA under the MOU and task plan PP-3664-9 concerning combating terrorism research and development and USA Departments of Defence; and the Advance Queensland funding initiative to establish The Translational Research Institute (TRI) Innovation and Translation Centre (IAT Centre) in co-operation with Siemens Healthcare.

Acknowledgments

We thank Dr Saad Ramadan PhD for assistance with the COSY code for each version of operating system; Professor Alexander McFarlane FRACP, Centre for Traumatic Stress Studies in Adelaide, for his support and assistance in establishing the correct evaluations for the PTSD cohort; Julie Mastrodomenico for undertaking the early psychological evaluations and Valerie Graves MM for the initial management of the program. Thank you to Psychiatrist Alexander Murray and Psychologist Roger Peters for their early help with recruitment.

Chapter 5

2D *in-vivo* L-COSY spectroscopy identifies neurometabolite alterations in multiple sclerosis

Scott Quadrelli, Karen Ribbons, Jameen Arm, Oun Al-iedani, Jeannette Lechner-Scott, Rodney Lea, Saadallah Ramadan.

Abstract

We have applied *in-vivo two*-dimensional (2D) Localised COrrelation SpectroscopY (2D L-COSY), in relapsing remitting multiple sclerosis (RRMS) to identify novel biomarkers to monitor disease activity in normal appearing brain parenchyma.

2D L-COSY MRS spectra was prospectively acquired from the posterior cingulate cortex (PCC) in 45 stable RRMS patients undergoing treatment with Fingolimod and 40 age and sex-matched healthy control participants (HC). Average metabolite ratios and clinical symptoms including, disability, cognition, fatigue and mental health parameters were measured and compared using parametric and non-parametric tests. Whole brain volume and MRS voxel morphology were evaluated using SIENAX and SPM LST toolbox.

Despite the mean whole brain lesion volume being low in this RRMS group (6.8mls), a significant reduction in PCC metabolite to tCr ratios were identified for multiple N-acetylaspartate (NAA) signatures, gamma-aminobutyric acid (GABA), glutamine and glutamate (Glx), threonine and isoleucine/lipid. Of the clinical symptoms measured, visuospatial function, attention and memory were correlated with NAA signatures, Glx and isoleucine/lipid in the brain.

2D L-COSY has the potential to detect metabolic alterations in the normal-appearing MS brain. Despite only examining a localised region, we could detect metabolic variability associated with symptoms.

Introduction

Multiple Sclerosis (MS) is a chronic immune-mediated demyelinating condition. Disease severity is monitored using clinical symptoms and magnetic resonance imaging (MRI) of the brain. In recently published guidelines for the management of MS cases, brain and spinal cord MRI have a prominent role in the diagnosis and follow up of dissemination in time and space of central nervous system involvement (1). There is increased reliance on MRI in the clinical management of MS, however as a primary outcome measure of efficacy in new therapeutic trials, MRI biomarkers are limited. There is a clinico-radiological paradox, such that there can be a poor association between clinical findings and radiological disease severity (Barkhof 2002, Chard and Trip 2017, Healy, Buckle et al. 2017). Next to multifocal white matter lesions, other contributing pathological features remain unrecognised due to the limitation of conventional MRI protocols (such as T1 and T2 weighted imaging) to detect CNS changes, including functional alterations in normal appearing brain matter (NABM). Changes in NABM, gray matter lesions (Geurts, Reuling et al. 2006) and neurodegeneration, lead eventually to brain atrophy and accumulating disability during the course of the disease (Bermel and Bakshi 2006,

Roosendaal, Bendfeldt et al. 2011). In addition, following the explosion in the number of new disease modifying therapies available for the treatment of MS in the last decade, it has become essential to better understand the underlying pathological changes associated with disease progression and thereby develop new meaningful imaging biomarkers to radiologically evaluate the clinical efficacy of treatments.

Total brain volume and atrophy have been correlated with overall disability status (Miller, Lublin et al. 2018), while gray matter volume (Roosendaal, Bendfeldt et al. 2011, Shiee, Bazin et al. 2012) has a strong predictive value with both physical disability and cognitive impairment in MS. Other novel MRI metrics enable metabolic changes in the MS brain to be explored and have the potential to provide biomarkers of disease progression. Proton magnetic resonance spectroscopy imaging (H^1-MRS) is a non-invasive technique allowing an evaluation of cerebral metabolites altering functional processes in the MS brain (Narayana 2005, Londono and Mora 2014). Studies conducted to date have been able to demonstrate metabolic changes occurring across multiple regions in the MS brain and that these alterations are not restricted to active white matter lesions, but also occur in regions of NABM (Fleischer, Kolb et al. 2016). By far the most common metabolite evaluated by MRS in MS to date has been N-acetylaspartate (NAA), with reduction in NAA content associated with axonal loss, neuronal damage and mitochondrial dysfunction (Steen, D'Haeseleer et al. 2013). Levels of NAA have been correlated with disability status (Khan, Seraji-Bozorgzad et al. 2017) and disease course (Aboul-Enein, Krssak et al. 2010, Obert, Helms et al. 2016) and with the magnitude of change differing between brain regions (Geurts, Reuling et al. 2006) as well as between lesions and NABM (Fleischer, Kolb et al. 2016). NAA levels have also been associated with the efficacy of MS therapies (Yetkin, Mirza et al. 2016, Khan, Seraji-Bozorgzad et al. 2017).

Conventional H^1-MRS is limited by the number of metabolites that can be quantified, due to overlapping resonances from the neurotransmitters such as glutamate, glutamine and gamma-aminobutyric acid (GABA), making evaluations of their potential role in MS difficult. For example, GABA has been proposed as a marker of neurodegeneration, however, it cannot be reliably quantified by conventional MRS (Cawley, Solanky et al. 2015). An alternative approach is two-dimensional (2D) MRS, which compensates for the limitations of conventional MRS, as it allows composite or overlapping resonances from 1D spectra to be separated out. In conventional 1D spectroscopy, intensity (y-axis) is plotted against frequency (x-axis), whereas in 2D spectroscopy, intensity is plotted against two frequency variables (Keeler 2010). The introduction of 2D in vivo spectroscopy has allowed researchers to make unambiguous metabolite assignments that previously could not have been made using 1D spectroscopy

(Thomas, Yue et al. 2001, Ramadan, Ratai et al. 2010, Mountford, Quadrelli et al. 2015) with 2D localised COrrelation SpectroscopY (2D L-COSY) being shown as a reliable method for in vivo detection of brain metabolites (Arm, Al-iedani et al. 2018).

We hypothesized that 2D spectroscopy would yield additional metabolic information in MS, not available using conventional MRS. The aim of the current work is to identify neurochemical differences in the NABM of clinically stable relapsing remitting MS patients, compared to age and sex-matched healthy individuals, using 2D L-COSY. We went on to determine if any of the metabolites identified were associated with clinical symptoms including disability status, cognitive function, mood status and fatigue. We also compared associations between features derived from other MRI approaches, including brain volume estimates and lesion load with clinical outcomes, thereby evaluating the benefits of obtaining additional information regarding alterations in metabolic species in the MS brain by 2D L-COSY.

Methods

Subject

Patients were prospectively recruited from the Multiple Sclerosis outpatient clinic at John Hunter Hospital, Newcastle, Australia from December 2014 to June 2017. Forty-five (45) RRMS patients and forty (40) age and sex matched control subjects (HC) were studied. Participants were considered age-matched if they were within ± 2 years of age to patients, with age at the time of scanning calculated according to date of birth. Patients were eligible if they had a confirmed diagnosis of RRMS, were aged between 18–65 years, had an Expanded Disability Status Scale (EDSS) score from 1–4 (able to walk a minimum of 500m) and were currently undergoing immunomodulatory therapy with Fingolimod. Patients were excluded if they had a comorbid diagnosis of other neurological or psychiatric conditions; impaired capacity to consent; any contraindication to MRI scanning or treatment with glucocorticoids within the last three months. HC were recruited from the Hunter Medical Research Institute (HMRI) research registry. HC were included if they were aged between 18–65 years and had no prior history of neurological or psychiatric disease. Participants were excluded if they had impaired capacity to consent; any contraindication to MRI scanning or were receiving opiates, antipsychotics or benzodiazepines. Demographic data and clinical histories were obtained at the time of study enrolment. Written informed consent was obtained from all participants prior to study commencement and the research protocol was approved by the Hunter New England Local Health District human research ethics committee.

Clinical Assessments

MS patients were examined by a neurologist and their disability status was evaluated using EDSS (Kurtzke 1983). All EDSS evaluations were undertaken by a neurologist who had undertaken appropriate Neurostatus certification training. The Multiple Sclerosis Severity Score (MSSS) was calculated from the EDSS and duration of disease for each patient using the algorithms provided by Roxburgh et al. 2005 (Roxburgh, Seaman et al. 2005). Study participants were assessed for cognitive performance using the Audio Recorded Cognitive Screen (ARCS), which is a valid and reliable instrument for administering neuropsychological tests of cognitive function to unsupervised individuals. The ARCS assess performance in the domains of memory, verbal fluency, language (object naming), visuospatial function and attention with elements from each domain score then used to derive an overall 'global' cognitive performance score. The Symbol Digit Modalities Test (SDMT) was undertaken concurrently as a measure of attention and information processing speed presented in the visual modality. The mental health status of participants was assessed using the short version of the Depression Anxiety Stress Scales (DASS-21). Higher scores were indicative of higher levels of depression, stress and anxiety. All scores, derived from the 21-point scale, were multiplied by 2 to enable comparison to the full 42-point scale DASS and determine clinical cut offs for symptom severity. Fatigue status was determined using the Modified Fatigue Impact Scale (MFIS), a modified form of the Fatigue Impact Scale. The questionnaire was based on items derived from interviews with MS patients concerning how fatigue impacts their lives. This instrument provided an assessment of the effects of fatigue in terms of physical and cognitive functioning.

MRI protocol

All scans were performed on a 3T Prisma (Siemens, Erlangen, Germany, software version VE11C) with a 64-channel head and neck coil (Siemens, Erlangen).

Structural Imaging

All participants underwent structural imaging that included: 3D T1-weighted magnetization-prepared rapid gradient-echo (MPRAGE) sequence (TR/TE/TI=2000/3.5/1100 ms, flip angle=7°, field of view=256x256 mm, voxel size 1x1x1mm^3, IPAT=2, acquisition time 4:48 minutes); and T2 fluid attenuated inversion recovery (FLAIR) (TR/TE/TI=5000/386/1800 ms, echo train duration=858ms, field of view=256 × 256 mm^2, with spatial resolution of 1×1×1 mm^3, IPAT=3, acquisition time 4:12 minutes).

2D L-COSY MR Spectroscopy

A 3D T1 MPRAGE was reconstructed in the sagittal and coronal planes with 2mm slice resolution for accurate localization of the voxel. Normal appearing brain matter (NABM) in the posterior cingulate cortex (PCC), composed of white and grey matter, was chosen for examination as shown in Figure 14. The PCC was chosen as it is a highly connected and metabolically active brain region (Leech and Sharp 2014), involved in learning and memory (Mountford, Quadrelli et al. 2015), has a favourable location for magnetic field shimming and is relatively insensitive to motion during spectral acquisition.

2D L-COSY was acquired using the following acquisition parameters: 96 increments and 8 averages per increment; RF carrier frequency at 2.0 ppm; TR 1.5 s; water suppression using WET; 96 t1 increments; with 8 averages per increment; PCC voxel size 3x3x3 cm^3, acquired vector size 1024 points; acquisition time 512 ms; spectral width in F2 2000 Hz and spectral width in F1 1250 Hz (0.8 ms increment size), total acquisition time of 19 minutes. Additional detail can be found in Ramadan et al (Ramadan, Andronesi et al. 2011).

Localised shimming was undertaken by adjustment of zero- and first-order shim gradients using the automatic B0 field mapping technique supplied by the vendor (Siemens AG) followed by manual adjustment of accessible shim gradients to achieve a resulting magnitude peak width of water at half-maximum of 15 Hz or less.

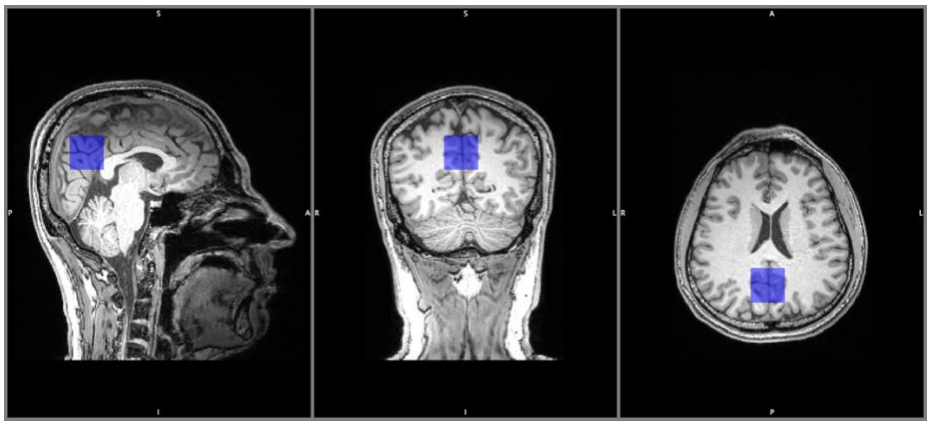


Figure 14- Representative voxel location in the PCC, in a healthy control participant.

2D L-COSY Quantification

All participants had satisfactory quality data for analysis. Raw 2D L-COSY data were transferred to MATLAB (2015b) (MathWorks 1984-2014) for signal combination from multiple elements followed by row concatenation into a 2D matrix. Felix, a commercial 2D spectral processing software (Accelrys Felix

NMR 2007), was used for spectral processing and analysis. The total creatine methyl diagonal resonance, at 3.02 ppm, was used as an internal chemical shift reference in F1 and F2. Absolute quantification of tCr has been performed separately using 1D spectroscopy, by this group and found to be stable in NABM in RRMS, confirming tCr (F2: 3.02 – F1: 3.02 ppm) was an appropriate L-COSY internal reference in RRMS. All 'cross' or off-diagonal peaks were denoted with F2 – F1 in ppm units. Several species with multiple cross peaks, such as glutamine and glutamate (Glx), NAA and lipid, were also summed together and compared. The processing parameters used were: F2 domain (skewed sine-squared window, 2048 points, magnitude mode), F1 domain (sine-squared window, linear prediction to 96 points, zero-filling to 512 points, magnitude mode). No additional water removal was applied, as water was sufficiently suppressed during acquisition. The volumes of cross peaks, or diagonal resonances, were evaluated using Felix software described above, and care was taken to ensure that the interrogated volume was the same in all 2D spectra, using a peak template with fixed chemical shift values.

Whole brain volume and WM lesion quantification:

FLAIR hyperintensities were segmented using the lesion growth algorithm (Schmidt, Gaser et al. 2012) as implemented in the LST toolbox version 2.0.6 (www.statistical-modelling.de/lst.html) for SPM. The algorithm first segments the T1 images into the three main tissue classes: cerebrospinal fluid, grey-matter and white-matter (CSF, GM and WM). This information is then combined with the co-registered FLAIR intensities in order to calculate lesion belief maps. By thresholding these maps with a pre-chosen initial threshold (κ) an initial binary lesion map was obtained which was subsequently grown along voxels that appear hyperintense in the FLAIR image, resulting in a lesion probability map. The initial κ threshold was selected by iterating κ and performing a visual inspection, for this data set a κ value of 0.1 was selected. A binary lesion mask was created for each participant using a threshold of 0.5. Hypointense lesions on the T1 MPRAGE were determined using the binary lesion mask and filled with intensities similar to voxels not contained within a lesion (referred to as 'lesion filling'), using the LST toolbox (Schmidt, Gaser et al. 2012). Lesion filling was performed to improve volume measurements and prevent errors in partial volume segmentation (Gelineau-Morel, Tomassini et al. 2012). Partial volume segmentation of the lesion filled T1 structural image was segmented using FSL FAST (Battaglini *et al.*, 2012) after brain extraction.

Brain tissue volume, normalised for subject head size, was estimated with SIENAX (Smith, Zhang et al. 2002), part of FSL (Smith, Jenkinson et al. 2004). SIENAX extracts brain and skull images from the single whole-head input data (Smith 2002). The brain image was then affine-registered to MNI152 space

(Jenkinson and Smith 2001, Jenkinson, Bannister et al. 2002) (using the skull image to determine the registration scaling); this was done to obtain the volumetric scaling factor, then used as normalisation for head size. Next, tissue-type segmentation with partial volume estimation was carried out (Zhang, Brady et al. 2001) in order to calculate total volume of brain tissue, including separate estimates of volumes of grey matter, white matter, peripheral grey matter and ventricular CSF.

Statistical Analysis

Differences between group means for clinical data, 2D metabolites and neuroimaging metrics were determined using independent-samples t-tests for means. Bivariate correlations among quantitative test variables were performed using Pearson's r tests. Since some of the variables tested deviated from parametric assumptions, we also performed non-parametric equivalent tests for mean differences and bivariate correlation i.e. Mann-Whitney U test and Spearman's rho tests, respectively. These tests were performed as a partial guard against false positives and to assist in the interpretation of the parametric test results. Since this study was largely exploratory in nature and consisted of multiple test variables we chose to use a relaxed significance threshold of 0.05 and interpret results accordingly.

Results

Participant demographics and characteristics

The RRMS patient cohort was predominantly female (73%) with an average age of 43.4 years. Average disability status was mild (mean EDSS=2) with an average duration of disease of 8 years. Only patients who had been undergoing treatment with the disease modifying therapy Fingolimod (Gilenya) for at least 6 months were included in the study. The average treatment duration for the patient cohort at the time of study assessments was 2 years (Table 9). The patient cohort had a poorer performance on both the ARCS and SDMT cognitive assessment tasks compared to age and sex matched HCs. The overall ARCS score, fluency and visuospatial domain scores were lower in the MS cohort, together with processing speed and attention, as measured by the SDMT. Self–reported ability to perform routine tasks (FAQ) was also worse in MS patients compared to HCs. Mood symptoms were also more pronounced in the MS cohort with 2 fold higher levels of depression and anxiety and 1.7 fold higher scores for stress compared to the HC group. The MS cohort also reported higher levels of fatigue ($p = <0.001$) than HCs with physical and cognitive fatigue scores increased by 3 and 2 fold ($p = <0.001$ for both indices) respectively.

Table 9 - Participant Demographic and Clinical Features

	Healthy Control	RRMS	P - value
N	40	45	-
Age (years)	42.7 ± 1.4	43.4 ± 1.4	0.71
Female (%)	63	73	-
Disease Duration (years)	N/A	7.9 ± 0.9	-
EDSS	N/A	2.0 ± 0.2	-
MSSS	N/A	3.1 ± 0.3	-
Memory	94.8 ± 2.3	88.0 ± 3.7	0.14
Fluency	96.0 ± 2.9	84.7 ± 2.7	0.005*M-W-U
Visuospatial	102.8 ± 0.6	99.8 ± 0.7	0.01*M-W-U
Language	9.13 ± 4.2	87.5 ± 3.3	0.5
Attention	99.8 ± 2.5	94.3 ± 2.0	0.07
Total ARCS	94.6 ± 2.7	86.8 ± 2.8	0.05*M-W-U
SDMT	59.1 ± 1.9	50.9 ± 1.6	0.00*M-W-U
FAQ	0.7 ± 0.3	3.7 ± 0.9	0.004*M-W-U
Depression	3.2 ± 0.7	7.5 ± 1.4	0.008*M-W-U
Anxiety	3.1 ± 0.8	6.7 ± 1.2	0.013*M-W-U
Stress	7.7 ± 1.2	13.0 ± 1.5	0.01*M-W-U
Total DASS	14.1 ± 2.4	26.4 ± 3.4	0.005*M-W-U
Physical Fatigue	5.8 ± 0.9	18.0 ± 1.4	<0.001*M-W-U
Cognitive Fatigue	7.7 ± 1.1	16.3 ± 1.4	<0.001*M-W-U
Total Fatigue	13.5 ± 1.8	34.4 ± 2.7	<0.001*M-W-U

EDSS: Expanded Disability Severity Scale, MSSS: Multiple Sclerosis Severity Scale, ARCS: Audio Recorded Cognitive Screen, SDMT: Symbol Digit Modalities Test, FAQ: Functional Assessment Questionnaire, DASS: Depression Anxiety and Stress Scale.
All data are expressed as mean ± standard error of the mean unless otherwise indicated.

***M-W-U Indicates significance (P≤0.05) using Mann Whitney U tests.**

2D L-COSY

A typical 2D L-COSY spectrum for a healthy control volunteer and a RRMS participant is shown in Figure 15. A summary of the statistically significant neurochemical differences is shown in Table 10. A statistically significant reduction in metabolite to tCr ratios was identified for multiple NAA signatures (labelled NAA I, NAA III and NAA IV), N-acetylaspartylglutamate (NAAG), Glx, GABA, threonine and isoleucine / lipid. Additionally, there was a significant reduction in the summed cross peaks for NAA and Glx. Summed lipids were increased (18%) in the RMMS group when compared to healthy controls,

that trended towards significance (p=0.054). No significant difference was identified in the choline containing compounds, lactate, macromolecules or myo-inositol.

Table 10 - Metabolite levels that significantly differ in the posterior cingulate cortex between healthy subjects and RRMS using 2D L-COSY

Metabolite	Average Chemical Shift (F2-F1) ppm	Healthy Control	RRMS	% Change	P-value
NAA	2.00 – 2.01	135.0 ± 1.1	129.0 ± 1.1	-5%	<0.0001*M-W-U
NAA I	4.36 – 2.55	20.3 ± 0.3	18.7 ± 0.3	-8%	<0.0001*M-W-U
NAA III	2.62 – 2.62	32.0 ± 0.4	29.9 ± 0.4	-8%	0.002*M-W-U
NAA IV	2.47 – 2.67	8.1 ± 0.1	7.7 ± 0.1	-6%	0.002*M-W-U
NAA $_{amide}$	7.81 – 7.81	1.0 ± 0.1	0.9 ± 0.03	-17%	0.027*M-W-U
NAA $_{aspartate\ moiety}$	4.30 – 4.30	5.7 ± 0.1	5.4 ± 0.4	-5%	0.51*M-W-U
NAAG $_{aspartyl\ moiety}$	2.76 – 2.47	1.8 ± 0.05	1.6 ± 0.04	-9%	0.02 *M-W-U
NAA sum	-	204.5 ± 1.6	193.9 ± 1.6	-6%	<0.0001*M-W-U
Threonine	3.27 – 3.62	1.3 ± 0.1	1.2 ± 0.04	-11%	0.062*M-W-U
GABA I	2.27– 1.98	5.7 ± 0.1	5.4 ± 0.1	-5%	0.002*M-W-U
GABA	2.28 – 2.28	7.8 ± 0.1	7.4 ± 0.1	-5%	0.006*M-W-U
Glx I	2.40 – 2.09	4.9 ± 0.1	4.7 ± 0.1	-6%	0.005*M-W-U
Glx sum	-	18.1 ± 0.2	17.4 ± 0.2	-4%	0.015*M-W-U
Glx upper	3.76 – 2.09	9.2 ± 0.1	8.8 ± 0.1	-4%	0.038
Glx lower	2.10 – 3.75	8.9 ± 0.1	8.5 ± 0.1	-5%	0.037*M-W-U
Total Lipids	-	93.4 ± 3.2	113.6 ± 9.3	18%	0.054
Isoleucine / Lipid	1.39 – 1.95	1.4 ± 0.1	1.2 ± 0.1	-16%	0.028*M-W-U
Macromolecules	0.92 – 2.06	2.2 ± 0.1	2.2 ± 0.1	1%	0.9

All data are expressed as mean + standard error of the mean unless otherwise indicated.
***M-W-U** **Indicates significance (P<0.05) using Mann Whitney U tests.**

NAA: N-acetylaspartate; NAA I-IV: additional N-acetylaspartate signatures; NAAG: N-acetylaspartylglutamate; NAA sum: sum of NAA cross-peaks; GABA: gamma-aminobutyric acid; Glx: glutamine and glutamate cross-peaks; Glx sum – contains the above and below the diagonal cross-peaks summed.

Whole brain and voxel characteristics

The spectroscopy voxel was comprised of 37% WM, 51% GM and 13% CSF. There was no significant difference in the partial volume fractions within the SVS voxel between the RRMS and control groups, as shown in Table 11.

There was a (-3%) significant reduction in whole brain volume in RRMS compared to HC (Table 11). The RMMS group had an increased ventricular volume (28%), in keeping with atrophic change and a reduction in WM volume (%5), when compared to healthy controls, these differences were significant with only parametric (t-test) means testing. There was no significant difference in the total or peripheral grey matter volumes. On average RRMS participants had a total T2 FLAIR lesion volume of 6.7mL.

Table 11 – Comparison of whole brain volumes and partial volumes within the MRS voxel.

Volumetric Measure	RRMS (N=45)	HC (N=40)	Difference (%)	P - value
Voxel CSF (%)	12.62 ± 0.75	11.89 ± 0.79	6%	0.64
Voxel GM (%)	50.46 ± 0.57	50.91 ± 0.46	-1%	0.11
Voxel WM (%)	36.92 ± 0.61	36.82 ± 0.75	0%	0.67
WBV (mL)	1572.5 ± 14.3	1617.5 ± 13.7	-3%	0.03*M-W-U
WM (mL)	756.1 ± 6.7	790.9 ± 7.0	-5%	0.001
GM (mL)	816.4 ± 9.1	826.6 ± 8.7	-1%	0.4
pGM (mL)	658.2 ± 7.4	665.7 ± 7.4	-1%	0.5
CSF (mL)	41.2 ± 2.8	29.6 ± 1.2	28%	0.001
Mean lesion volume (mL)	6.7	n/a	n/a	n/a

WBV: normalised whole brain volume, WM: normalised white matter volume, GM: normalised grey matter volume, pGM: normalised cortical grey matter, CSF: normalised ventricular volume.

***M-W-U* Indicates significance (P<0.05) using Mann Whitney U tests.**

Correlation of metabolites with clinical and volumetric measures

Multiple correlations between the NAA chemical finger prints, clinical and demographic measures were identified (Table 12). Specifically, NAA-I was strongly, and NAA-III and Glx-I cross peaks were moderately negatively correlated with disease duration. As expected NAA signatures were negatively correlated with age. Macromolecules were the only species correlated with disease severity (MSSS and EDSS).

Multiple correlations between molecular species and cognitive function were identified. Cognitive assessment (total ARCS) was negatively correlated with summed Glx cross peaks, and the isoleucine/lipid cross peak (Pearson's correlation: -0.30 and -0.39. p=0.046 and 0.008 respectively). Visuospatial cognitive function was negatively correlated with NAA-I, Glx- I and the NAAG aspartate moiety cross peak (Pearson's correlation: -0.41; -0.39; -0.33. p=0.005; 0.007; 0.025 respectively).

Attention was moderately negatively correlated with the isoleucine/lipid cross peak (Pearson's correlation: -0.39. p=0.004).

NAA signatures were significantly positively correlated with whole brain volume (WBV), but also with grey matter volume (GMV), and white matter volume (WMV) individually. CSF volume was moderately negatively correlated with NAA signatures. The Glx I cross peak was moderately positively correlated with GM volume. Subcortical grey matter volumes were positively correlated with the same NAA signatures, that were significantly correlated with total GM volume. A single cross peak, isoleucine/lipid, was significantly and positively correlated with the total lesion number, but not with lesion volume.

Correlation of clinical and volumetric measures

Of the brain volume measurements GMV and total lesion volume (TLV) were the most closely associated with cognitive function and disease severity. None of the volumetric parameters tested predicted fatigue and only the combined score of depression, anxiety and stress correlated weakly with WMV. TLV was strongly negatively correlated with WBV and GMV (Pearson's correlation-0.64 and -0.59. p=<0.001 for both).

Table 12 - Significant correlations between 2D L-COSY metabolite levels in the posterior cingulate cortex, whole brain volumes and lesion load of RRMS patients vs. clinical symptoms and brain volumes.

Clinical Measure	Metabolite	Correlation	P - value
Age	NAA I	-0.52	<0.001*
	NAA sum	-0.43	<0.001*
	Glx I	-0.41	<0.005*
	cGM	-0.49	<0.001*
MS disease duration	NAA III	-0.54	<0.001*
	NAA I	-0.32	<0.03*
	Glx I	-0.40	0.006*
	WBV	-0.48	<0.001*
	CSF	-0.50	<0.001*
MSSS	Macromolecules	0.42	0.004*
EDSS	Macromolecules	0.31	0.04*
	pGM	-0.49	<0.001*
	TLV	0.46	0.001*
Total DASS	WM	0.34	0.02
Cognitive Function			
Total ARCS	Total Glx	-0.30	0.046*
	Iso-leucine / Lipid	-0.39	0.008*
	TLV	-0.33	0.03
Visuospatial	NAA I	-0.41	0.005*
	Glx I	-0.39	0.0074 *
	$NAAG_{aspartate}$	-0.33	0.025*
Attention	Iso-leucine / Lipid	-0.39	0.004 *
	TLV	-0.41	0.005
Memory	Iso-leucine / Lipid	-0.44	0.002*
Fluency	WM	0.31	0.04
	TLV	-0.35	0.02
SDMT	WBV	0.34	0.02
	GM	0.34	0.02
	pGM	0.39	0.008
	TLV	-0.4	0.006
Brain Volumes			
WBV	NAA	0.4	0.006*
	NAA sum	0.43	0.003*
	TLV	-0.64	<0.001*
GM	NAA	0.40	0.006*
	NAA IIII	0.45	0.002*
	NAA IV	0.40	0.007*
	NAA sum	0.46	0.002*
	Glx I	0.36	0.02*
	TLV	-0.59	<0.001*

WM	NAA	0.31	0.04*
	TLV	-0.37	0.013
CSF	NAA III	-0.37	0.013*
	NAA sum	-0.30	0.048*
	TLV	0.38	0.001
TLN	Iso-leucine / Lipid	0.44	0.003*

ARCS: Audio Recorded Cognitive Screen; cGM: Cortical Grey Matter; CSF: Cerebrospinal Fluid Volume; EDSS: Expanded Disability Status Scale; GM: Gray-Matter volume; MSSS: Multiple Sclerosis Severity Scale; SDMT: Symbol Digit Modalities Test; TLN: Total Lesion Number; WM: White Matter volume.

Correlations are made between 2D L-COSY metabolite levels and demographics, clinical symptoms and partial brain volumes in the RRMS (N=45) group.

Pearson's correlations are shown. * Indicates significance ($P \leq 0.05$) using Spearman's test.

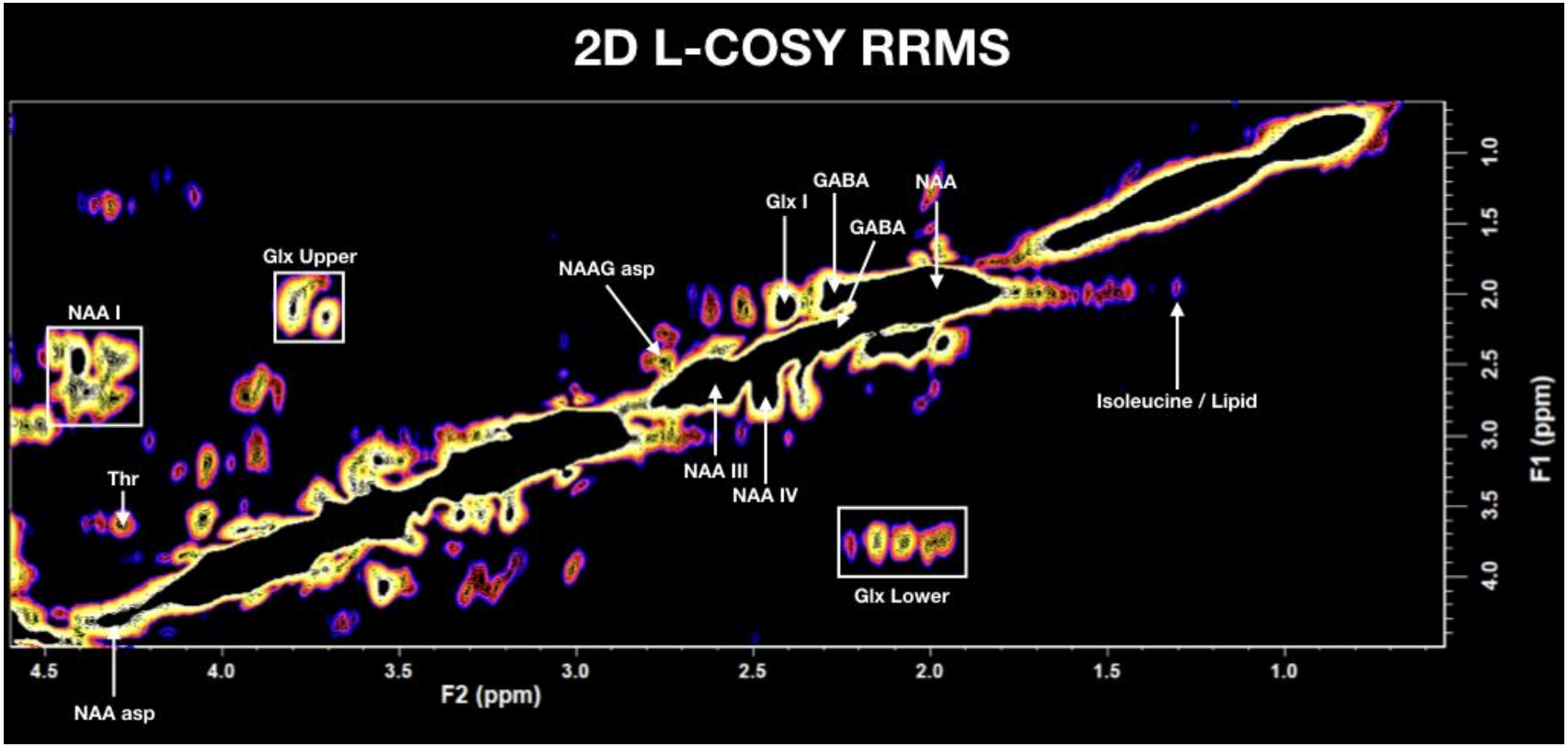


Figure 15 - In vivo L-COSY of a healthy volunteer (PCC) acquired at 3T using a 64 channel head and neck coil; voxel size 30x30x30 mm3, increment size 0.8ms, increments 96, 8 averages per increment, TR 1.5 sec, total experimental time 19 min, acquired vector: 1024 points, acquisition time: 512 ms, spectral width in F2: 2000 Hz, spectral with in F1: 1250 Hz. Abbreviations: NAA: N-acetylaspartate; NAA I-IV: additional N-acetylaspartate signatures; NAA asp: NAA aspartate moiety; NAAG: N-acetylaspartylglutamate; GABA: gamma-aminobutyric acid; Glx: glutamine and glutamate cross-peaks; Thr: Threonine.

Discussion

Our RRMS patient cohort was comprised of patients who were clinically stable and currently undergoing treatment with Fingolimod. The lesion load was low in these patients and current disability status was mild, at EDSS 2. The MRS voxel, located in the PCC and evaluated by 2D L-COSY, predominantly contained normal appearing white and gray matter. Whilst disability status was mild, there was evidence of impaired cognitive function and higher levels of mood and fatigue symptoms in RRMS compared to matched healthy subjects. Despite a stable disease status, we were able to detect metabolic alterations in the MS brain in NABM, which was comparable to that observed in other studies (Geurts, Reuling et al. 2006) (Richards 1991, Kapeller, McLean et al. 2001, Tisell, Leinhard et al. 2013, Muhlert, Atzori et al. 2014). These differences cannot be attributed to morphological differences in the region of interest between control and MS patients, as there was no difference in the WM, GM or CSF MRS voxel composition between groups. The lack of a strong association between whole brain lesion volume and the presence of clinical symptoms in our cohort, highlights the clinico-radiological paradox and supports a potential use of MRS to identify other contributing pathological factors in MS.

Using 2D L-COSY we identified a reduction in multiple NAA metabolic fingerprints and the summed glutamine and glutamate metabolic signatures. 2D L-COSY also enabled the identification of MS-specific changes in additional metabolites, not quantifiable using other techniques, such as GABA, that historically requires an edited pulse sequence for accurate quantification (Mescher, Merkle et al. 1998).

A reduction in NAA has been shown by others as the most consistent metabolic abnormality found in NABM in the MS brain (Rovira and Alonso 2013). The metabolic fingerprints of NAA in the PCC were significantly correlated with reductions in all whole brain volumetric measures, with a strong correlation with WBV. A significant reduction in the WBV was identified in the RRMS group (3%), however we were able to detect a greater reduction in NAA fingerprints (NAA sum: 6 % and up to -17% for the NAA amide moiety) in the PCC of the MS group, despite this being detected in NABM. These findings suggest that NAA may be a more sensitive marker of atrophic change, when compared to volumetric measures. NAA levels were also associated with disease duration, which may also be linked with increased brain atrophy during the course of the disease (Eshaghi, Marinescu et al. 2018). Longitudinal studies are required to further clarify this finding and to determine if techniques, such as whole brain NAA can further increase the sensitivity and clinical usefulness of MRS.

We identified a small but statistically significant reduction in Glx in NABM, that was negatively associated with cognitive function (total ARCS), specifically in the visuospatial domain. Additionally, Glx

cross peaks were found to be negatively correlated with disease duration. This finding supports the work of Muhlert et. al. (2014), who identified reductions in glutamate in the cingulate and parietal cortices, associated with reduced visuospatial memory in MS patients (Muhlert, Atzori et al. 2014). One possible explanation for this finding is reduced synaptic activity due to synaptic loss (Wegner, Esiri et al. 2006), which is also supported by findings in the aging brain (Kaiser, Schuff et al. 2005). This is further supported by the significant positive correlation that was identified between a Glx cross peak and whole brain GM volume.

GABA is the major inhibitory neurotransmitter in the brain. To our knowledge reductions in GABA have not been previously reported in NABM in the PCC in RRMS, however, a recent study found reduced GABA levels in secondary progressive patients in the hippocampus and the sensorimotor cortex, associated with reduced motor performance (Cawley, Solanky et al. 2015). In the current study, we did not find a correlation between GABA, brain volume or cognitive function, most likely due to the early disease stage of our cohort.

Isoleucine is an essential amino acid. This is the first *in-vivo* evidence of a reduction of isoleucine in the brain in multiple sclerosis. Supporting this finding, Hyun-Hwi et al. found reduced isoleucine in the CSF of patients with multiple sclerosis who were in relapse, when compared to patients in remission, using *in-vitro* MRS (Kim, Jeong et al. 2017). Isoleucine was associated with multiple measures of cognitive function, including total ARCS, attention and memory domains of cognitive function. Further work is required to confirm these findings, as the cross peak for Isoleucine is in a location where there is contamination from T1 noise and lipid.

A second amino acid, threonine, was found to be significantly reduced between RRMS patients and healthy controls. Threonine was not correlated with clinical symptoms or volume measures.

Despite a low whole brain lesion load, we did observe an overall 3% reduction in total brain volume and 5% loss in white matter content, with a reciprocal 28% increase in CSF content, compared to healthy age and sex matched controls. Lower brain volumes have been linked to an increase risk of disease progression rates and reduced effect of therapy in MS cases (Sormani, Kappos et al. 2017) and supports the notion that disease processes other than white matter demyelination are occurring in the MS brain. The rate of atrophy continues to be investigated as a potential marker of disease progression in MS (De Stefano, Stromillo et al. 2016) and indeed regional atrophy in gray matter structures, particularly the thalamus, have been shown to have a high correlation of cognitive performance in MS (Bergsland, Zivadinov et al. 2016). Although the current study was a cross-sectional design, from which we cannot

evaluate the level of atrophy, we do see a moderate positive association between WBV, cortical grey matter and GMV with cognitive function as, determined by the SDMT (Table 12).

Currently 2D L-COSY technology has some limitations, such as the high SNR requirement for adequate data quality, thus the time of acquisition is currently 19 minutes. Work is currently being undertaken by our group and others Thomas et al. (2014) to accelerate 2D L-COSY. Like with many SVS techniques, the 2D L-COSY voxel needs to be large (~27cm^3), limiting the accuracy with which brain regions can be interrogated.

This study has been carried out with a small numbers of participants (total n=85). Despite this, every effort has been made to control for confounding variables by age and sex matching the groups. On-going investigations are warranted, to undertake longitudinal evaluations of changes in metabolites to further appreciate the biochemical processes that underpin the changes in metabolite levels we observed, and assess the usefulness of these chemical entities as potential biomarkers for disease severity and progression.

Conclusion

Using 2D L-COSY, we were able to report on metabolites that were otherwise not available for analysis using 1D spectroscopy. Using 2D L-COSY we provide *in vivo* evidence of a reduction in multiple NAA signatures, GABA, Glx and isoleucine /lipid in normal appearing brain matter of RRMS patients. The additional metabolic information provided may be helpful in the future to unlock the pathophysiology of RRMS and identifying biomarkers for RRMS, disease severity and clinical progression.

Acknowledgments

We would like to acknowledge all the study participants that took part in this investigation.

Funding for this study provided by Novartis Pharmaceuticals Australia.

Chapter 6

Conclusion and Future Directions

Summary

This thesis describes the further development and application of *in-vivo* neuro 2D L-COSY, a 2D spectroscopy technique, to measure differences in brain biochemistry. In Chapter 2 I asked the question, "C*an 2D L-COSY be used to assign fucosylated glycans in the human brain in-vivo*?". This work was an extension of work originally performed by Lean et al (1991), where fucosylated glycans were first assigned *in-vitro* using a human colorectal cancer cell model. At that time fucose signalling was shown to be utilised by cancers to facilitate hematogenous metastasis and to be important in the inflammatory cascade, specifically white blood cell 'rolling' in response to infection. Using the evidence from this *in-vitro* work and after applying 2D L-COSY to many healthy controls we recorded spectra that indicated this spectral region, between F2: 3.95 - 4.50 ppm and F1: 0.90 - 1.70 ppm, to be populated with the same type of fucosylated molecules that were assigned *in-vitro*. Using 2D L-COSY we were able to successfully assign up to six Fucose-α(1-2)- galactose species in the human brain.

Post processing is important in magnetic resonance spectroscopy (MRS) and next I aimed to determine the best approach to perform partial volume correction of 1D and 2D MRS (Chapter 3). I successfully outlined and published a best practice methodology for performing partial volume correction from a (MRS) voxel, and automated the procedure to extract partial volume metrics and other data of interest such as white matter lesions in MS. The techniques developed in this chapter were utilised in the subsequent chapters, where 1D and 2D MRS were applied to posttraumatic stress disorder (PTSD) and multiple sclerosis (MS).

In Chapter 4 I asked, *"Can MRS be used to identify unique metabolic differences in the brains of PTSD patients when compared to healthy controls?".* Prior to undertaking my own study, I performed a systematic review of research evaluating PTSD using MRS. The review identified multiple brain regions implicated in the pathogenesis of PTSD, such as the amygdala, hippocampus, and cingulate cortex to focus on a few. The review also revealed that the most consistent MRS abnormality identified in PTSD is reduced N-acetylaspartate (NAA) in the anterior cingulate cortex (ACC) and hippocampus, predominantly without atrophic change, suggesting that MRS may be a more sensitive marker of early volumetric change. More recent studies have demonstrated evidence of glutamatergic dysfunction and changes in GABA, in the brains of PTSD patients, suggesting these neurotransmitters may have a role in the pathogenesis of PTSD.

We then went on to perform our own study using 1D MRS and 2D L-COSY in PTSD, where I asked the question, *"Can 2D L-COSY identify unique metabolic changes in the brains of PTSD patients, not seen*

using 1D MRS? ". After comparing PTSD participants to healthy controls we identified a reduction in absolute Inositol, Inositol:Cr, and Inositol:NAA in the PCC and increased Glu:tCr and Glx:tCr in the ACC using 1D spectroscopy. The finding of increased Glx in the ACC is supported by several other studies, and further raises the possibility that increased glutamate may result in excitotoxicity in this region (Meyerhoff, Mon et al. 2014, Pennington, Abe et al. 2014). The finding of reduced Inositol in the PCC has not been described before in PTSD. Inositol is thought to be a astroglial marker and a reduction, may be secondary to cell lysis (Harris, Choi et al. 2015).

Using 2D spectroscopy we were able to identify additional metabolic changes, not previously described using conventional spectroscopy in PTSD. Specifically, we recorded a reduction in total fucose and the fucosylated glycans, Fuc IV and VI in the PCC. Here I provide the first in-vivo evidence that specific fucose species are altered in PTSD, suggesting fucose may play a role in PTSD pathogenesis and symptomatology. Supporting this finding, Revest et al. (2010) found that blocking fucosylation of synapsin Ia and Ib, proteins that contain fucose in the highest concentration in the brain, inhibits the glucocorticoid mediated increase in stress related memories in the hippocampus (Revest, Kaouane et al. 2010). It is through this pathway that glucocorticoids enhance the memory of stress related events, it is possible that 2D L-COSY and the fucose region could be utilised to non-invasively monitor this pathway.

Using 2D L-COSY we also identified, in the PTSD cohort, a reduction in imidazole ('IMI-1' – from histamine, histadine and homocarnosine) and in the CH_3 lipid cross peak (F2: 1.37 – F1: 1.99 ppm), two species that cannot be quantified using 1D MRS. Levels of the 'IMI-1' metabolite were significantly positively correlated with hyperarousal symptoms (r=0.66, p=0.04), early evidence that 2D L-COSY may be able to provide clinically relevant biomarkers in PTSD.

Finally, in this thesis, I asked the question, *"Can 2D L-COSY be applied to the autoimmune demyelinating condition multiple sclerosis, to provide clinically relevant biomarkers to monitor disease severity and efficacy of treatments?".* We found a significant reduction in posterior cingulate cortex metabolite to total creatine ratios for multiple NAA signatures, gamma-aminobutyric acid (GABA), glutamine and glutamate (Glx), threonine and isoleucine/lipid. Of the clinical symptoms measured, visuospatial function, attention and memory were correlated with NAA signatures, Glx and isoleucine/lipid in the brain. Despite only examining a localised region, we could detect metabolic variability associated with symptoms in multiple sclerosis. Unlike in PTSD, no differences in fucosylated glycans were identified in the multiple sclerosis cohort examined.

Thus, the results from my thesis have provided an important step forward for understanding the in-vivo 2D spectroscopy in the brain. Using 2D L-COSY we were able to assign novel fucosylated glycans in the human brain in-vivo. Additionally, using 2D L-COSY I have demonstrated that there are unique chemical changes in the brain of patients with PTSD and MS, several of these metabolic changes are correlated with clinical metrics, and may be useful for disease monitoring in the future. Based on the findings of this thesis, I propose several projects and experiments that could be undertaken to advance 2D L-COSY and the field of in-vivo neurospectroscopy in general.

Future Directions

Further technical advancements required to translate 2D L-COSY into clinical radiology:

The acquisition time of 2D L-COSY is long, approximately 20 minutes, limiting the applicability of the technology in the clinical setting. The time of acquisition is a trade-off between signal to noise ratio (SNR), number of averages, number of increments and MRS voxel size. Currently the high SNR requirements of L-COSY dictate a large MRS voxel, normally 27cm^3, limiting the regions of the brain that can be accurately interrogated. For example, it is currently not possible to acquire 2D L-COSY data from the amygdala, due to its small size, without significant contamination from the adjacent hippocampus. One possible solution to accelerate data acquisition is non-uniform sampling, a technique that has been successfully applied in nuclear magnetic resonance spectroscopy. Future work is required to apply and translate these techniques to 2D L-COSY, with the aim of reducing the acquisition time to less than five minutes, to minimise patient movement and to allow other sequences to be acquired during a clinical scan.

Additional work is required to improve the pre- and post- processing of 2D L-COSY. Frequency drift correction has been shown to be an important step in the post processing of 1D MRS data (Rowland, Liao et al. 2017) and whilst drift may have a limited impact on spectroscopy acquired from the brain, it may be a significant issue for 2D MRS acquired from other regions such as the breast. In this work there is no drift correction performed as part of the post-processing pipeline of 2D L-COSY data, however this may provide SNR and peak width gains. Currently, chemical signatures are measured using manual peak picking, which is a useful technique when performing untargeted analyses with newly assigned metabolites. However, this method has limitations, due to the technical difficulty and room for interobserver variability. Additionally, there are metabolites such and glutamine and glutamate that still cannot be resolved using peak picking alone. One solution is peak fitting, using a basis set of known metabolites, as has been implemented for 1D spectroscopy in the commercial package 'LC-Model' (Provencher 2001). A fitting routine has previously been developed for 2D JPRESS (Schulte and Boesiger 2006, Wolf, Pujara et al. 2015), however there is no technique available for 2D L-COSY as yet. A fitting technique for 2D L-COSY would not only be helpful for the reliability and accuracy of quantification, glutamine and glutamate could be accurately deconvolved. I predict that measurement of metabolites such as glutamine and glutamate, using peak fitting and 2D L-COSY, will be more robust than those obtained from 1D MRS. In addition to fitting routines, future work is planned to use machine learning techniques to help separate clinical conditions using 2D L-COSY.

Fucoslyated glycans and their role in learning and memory:

Whilst we have made the assignment of fucoslyated glygans in the brain, it not currently known what these species relate to or their function in the brain. Synapsin is the most abundant glycoprotein in the brain, and therefore I hypothesise that the presence of fucose in the brain is secondary to the synapsin proteins (Murrey HE, Gama CI et al. 2006, Murrey, Ficarro et al. 2009). Synapsin is a synaptic vesicle associated protein thought to regulate synaptic transmission (Hilfiker, Pieribone et al. 1999). Mammalian genes code for three separate synapsin proteins, synapsins: I, II and III. Synapsin proteins have been shown to be clinically relevant in epilepsy, where mutations in synapsin I and II have been linked with epilepsy and schizophrenia due to lower expression (Dyck, Beyaert et al. 2011, Tan, Dyck et al. 2014) of synapsin II. Also, as described above synapsin Ia and Ib appear to play a role in the glucocorticoid enhancement of stressful memories (Revest, Kaouane et al. 2010). The first experiment that should be undertaken to determine whether the presences of fucose in the human brain is linked to synapsin proteins, is to acquire 2D spectroscopy data from samples of recombinant synapsin I, II and III proteins. This can be further extended to acquiring in-vivo spectroscopy data from synapsin knock-out mice, to determine how fucose changes in-vivo depending on which synapsin protein is deficient. Our group continues to explore how fucosylated glycans change as a result of disease and ageing, and recruitment is currently underway to measure fucose in an adolescent population.

Exploring the pathogenesis of PTSD using MRS and 2D L-COSY:

In this thesis I have provided in-vivo evidence of unique metabolic differences in the brains of patients with PTSD, using 2D L-COSY in a region not well associated with PTSD, the PCC. With future technical advances described above, L-COSY should be applied to different regions of the brain such as the hippocampus, a key region highly implicated in the pathogenies of PTSD. Additionally, advances in techniques such as accelerated, high resolution 3D chemical shift imaging, should be employed to quantify the metabolic changes in the amygdala in PTSD, a region that has not yet been interrogated using MRS.

Studying PTSD is challenging due to the high number of co-morbidities associated with the condition, such as alcohol use disorder, and the fact that patients are often taking medications to treat their condition. It is still not clear to what extent co-morbid conditions affect new imaging techniques, however researchers are advised to maintain the 'cleanest' cohorts possible to minimise the chance of bias.

Despite the cohort size limitation in the PTSD study presented in this thesis, we still found large statistically significant differences in metabolites. Future work should focus on increasing the cohort sizes to determine other metabolic differences that may not have reached significance in this smaller cohort. There is also a need for further prospective studies in the field of PTSD using MRS. These studies should aim to answer the following questions: does lower hippocampal volume predispose to PTSD?; do metabolic differences in the hippocampus proceed atrophy?; are there MRS differences in the dorsal ACC that predispose to PTSD ? and; are rostral ACC and insular cortex MRS differences acquired as part of the condition?.

Further, to identify useful clinical biomarkers RRMS using MRS and 2D L-COSY:

In this thesis I have provided evidence that 2D L-COSY can detect metabolic alterations in the normal appearing brain in RRMS. Despite only examining a localised region of the brain we were able to detect biochemical variations that were associated with symptoms. Ongoing studies are required to determine how metabolites vary with disease course, and to confirm if MRS and 2D L-COSY is a more sensitive marker of atrophy than conventional MRI. It is possible that whole brain techniques, such as 3D CSI will be useful in measuring metabolic changes in multiple regions and may yield novel clinically useful imaging biomarkers in MS.

Conclusions

Magnetic resonance imaging (MRI) is a widely adopted technique in current clinical and radiological practice, allowing for the accurate identification of anatomical structures and disease processes. However, conventional imaging has limitations when exploring the underlying biochemical change driving a disease process. MRS is a technique which allows for non-invasive evaluation of tissue metabolism, which may be disturbed as a result of pathology. MRS is therefore well-suited to the provision of additional information regarding disease processes where there are limited conventional imaging findings or a mismatch between imaging findings and symptoms. For these reasons MRS may be a key part of the field of 'Radiomics'. In my thesis we used the 2D *in-vivo* localised spectroscopy technique, 2D L-COSY, to assign up to six fucose α(1-2)- galactose species for the first time in the human brain. Fucosylated glycans have previously been shown in animal models to be important in learning, development and memory, and are contained in high concentration within synapsin proteins, a protein critical for vesicle transport. It is possible that the fucose identified using this technology is related to synapsin proteins and further work is planned to confirm this. In this thesis 2D L-COSY was applied to two clinical conditions, PTSD and MS, both of which require additional imaging biomarkers for

diagnosis, prognosis and monitoring of disease severity. Using the new fucosylated glycans assignment and 2D L-COSY, I was able to identify a reduction in total fucose and the fucosylated glycans Fuc-IV and Fuc VI in the posterior cingulate cortex in PTSD. This is the first in-vivo evidence of dysregulation of fucose in PTSD. Additionally, in the first study to be performed using 2D L-COSY in MS, I used this technology to measure metabolic differences in the normal-appearing brain in relapsing and remitting MS (RRMS). In MS I found a reduction in multiple NAA signatures, along with a reduction in GABA, Glx and iso-leucine. Signatures for NAA and Glx were negatively correlated with disease duration and multiple metabolic signatures, such as Glx and iso-leucine, were negatively correlated with cognitive function. No changes in fucosylated glycans were identified in the brain of multiple sclerosis patients.

Using 2D L-COSY I have identified multiple metabolic differences not previously described in PTSD and RRMS. Coupling the information derived from my thesis: future experiments will focus on the origin of fucose in the brain; how fucosylated glycans change in the brain as a result of pathology, such as chronic pain and mild traumatic brain injury and; how these glycans change with the ageing process. Additionally, my future work will focus on the use of machine learning techniques to separate disease states and to identify and validate biomarkers for disease severity, using data obtained from 2D L-COSY. Further work is also required to accelerate the speed of L-COSY acquisition, improve post processing technique and develop quantification techniques through peak fitting.

In this thesis, I provided extensive experimental evidence supporting 2D L-COSY as a valuable in-vivo spectroscopic technique, that can provide metabolic information not available from current MRS techniques.

References

Aboul-Enein, F., M. Krssak, R. Hoftberger, D. Prayer and W. Kristoferitsch (2010). "Reduced NAA-levels in the NAWM of patients with MS is a feature of progression. A study with quantitative magnetic resonance spectroscopy at 3 Tesla." PLoS One **5**(7): e11625.

Accelrys Felix NMR (2007). Felix NMR.

Admon, R., M. R. Milad and T. Hendler (2013). "A causal model of post-traumatic stress disorder: disentangling predisposed from acquired neural abnormalities." Trends Cogn Sci **17**(7): 337-347.

Alden, M., J. M. Besson and J. F. Bernard (1994). "Organization of the efferent projections from the pontine parabrachial area to the bed nucleus of the stria terminalis and neighboring regions: a PHA-L study in the rat." J Comp Neurol **341**(3): 289-314.

Allen, P. S. (1990). "In vivo NMR spectroscopy." Basic Life Sci **55**: 419-426.

Amano, T., S. Duvarci, D. Popa and D. Pare (2011). "The fear circuit revisited: contributions of the basal amygdala nuclei to conditioned fear." J Neurosci **31**(43): 15481-15489.

American Psychiatric Association (1994). Diagnostic and Statistical Manual of Mental Disorders. Washington, DC.

Amorapanth, P., J. E. LeDoux and K. Nader (2000). "Different lateral amygdala outputs mediate reactions and actions elicited by a fear-arousing stimulus." Nat Neurosci **3**(1): 74-79.

Arm, J., O. Al-iedani, S. Quadrelli, K. Ribbons, R. Lea, J. Lechner-Scott and S. Ramadan (2018). "Reliability of neurometabolite detection with two-dimensional localized correlation spectroscopy at 3T." Journal of Magnetic Resonance Imaging.

Ashburner, J., G. Barnes, C.-c. Chen, J. Daunizeau, R. Moran, R. Henson, V. Glauche and C. Phillips (2015). SPM12. London, Functional Imaging Laboratory.

Augustine, J. R. (1996). "Circuitry and functional aspects of the insular lobe in primates including humans." Brain Res Brain Res Rev **22**(3): 229-244.

Babor, T., J. De la Fuente, J. Saunders and M. Grant (1992). AUDIT: The Alcohol Use Disorders Identification Test: Guidelines for Use in Primary Health Care. Geneva, World Health Organization.

Barkhof, F. (2002). "The clinico-radiological paradox in multiple sclerosis revisited." Curr Opin Neurol **15**(3): 239-245.

Bauer, P., Elizabeth; Pare, D. (2016). Behavioural neuroscience of circuits involved in fear processing. Neurobiology of PTSD: From Brain to Mind. I. R. Liberzon, Kerry. New York, USA, Oxford University Press.

Becker, D. J. and J. B. Lowe (2003). "Fucose: biosynthesis and biological function in mammals." Glycobiology 13(7): 41R-53R.

Behar, K. L., D. L. Rothman, K. F. Petersen, M. Hooten, R. Delaney, O. A. Petroff, G. I. Shulman, V. Navarro, I. L. Petrakis, D. S. Charney and J. H. Krystal (1999). "Preliminary evidence of low cortical GABA levels in localized 1H-MR spectra of alcohol-dependent and hepatic encephalopathy patients." Am J Psychiatry 156(6): 952-954.

Bergsland, N., R. Zivadinov, M. G. Dwyer, B. Weinstock-Guttman and R. H. Benedict (2016). "Localized atrophy of the thalamus and slowed cognitive processing speed in MS patients." Mult Scler 22(10): 1327-1336.

Bermel, R. A. and R. Bakshi (2006). "The measurement and clinical relevance of brain atrophy in multiple sclerosis." Lancet Neurol 5(2): 158-170.

Binesh, N., K. Yue, L. Fairbanks and M. A. Thomas (2002). "Reproducibility of localized 2D correlated MR spectroscopy." Magn Reson Med 48(6): 942-948.

Blake, D. D., F. W. Weathers, L. M. Nagy, D. G. Kaloupek, F. D. Gusman, D. S. Charney and T. M. Keane (1995). "The development of a Clinician-Administered PTSD Scale." J Trauma Stress 8(1): 75-90.

Bonnelle, V., R. Leech, K. M. Kinnunen, T. E. Ham, C. F. Beckmann, X. De Boissezon, R. J. Greenwood and D. J. Sharp (2011). "Default mode network connectivity predicts sustained attention deficits after traumatic brain injury." J Neurosci 31(38): 13442-13451.

Boscarino, J. A. (2012). "PTSD is a risk factor for cardiovascular disease: time for increased screening and clinical intervention." Prev Med 54(5): 363-364; author reply 365.

Boulanger, Y., M. Labelle and A. Khiat (2000). "Role of phospholipase A(2) on the variations of the choline signal intensity observed by 1H magnetic resonance spectroscopy in brain diseases." Brain Res Brain Res Rev 33(2-3): 380-389.

Brabec, J., A. Rulseh, B. Hoyt, M. Vizek, D. Horinek, J. Hort and P. Petrovicky (2010). "Volumetry of the human amygdala - an anatomical study." Psychiatry Res 182(1): 67-72.

Brady, K. T., T. K. Killeen, T. Brewerton and S. Lucerini (2000). "Comorbidity of psychiatric disorders and posttraumatic stress disorder." J Clin Psychiatry 61 Suppl 7: 22-32.

Brand, A., C. Richter-Landsberg and D. Leibfritz (1993). "Multinuclear NMR studies on the energy metabolism of glial and neuronal cells." Dev Neurosci 15(3-5): 289-298.

Brant-Zawadzki, M., G. D. Gillan and W. R. Nitz (1992). "MP RAGE: a three-dimensional, T1-weighted, gradient-echo sequence--initial experience in the brain." Radiology 182(3): 769-775.

Brereton, I. M., G. J. Galloway, S. E. Rose and D. M. Doddrell (1994). "Localized two-dimensional shift correlated spectroscopy in humans at 2 Tesla." Magn Reson Med 32(2): 251-257.

Breslau, N., R. C. Kessler, H. D. Chilcoat, L. R. Schultz, G. C. Davis and P. Andreski (1998). "Trauma and posttraumatic stress disorder in the community: the 1996 Detroit Area Survey of Trauma." Arch Gen Psychiatry 55(7): 626-632.

Brown, S., T. Freeman, T. Kimbrell, D. Cardwell and R. Komoroski (2003). "In vivo proton magnetic resonance spectroscopy of the medial temporal lobes of former prisoners of war with and without posttraumatic stress disorder." J Neuropsychiatry Clin Neurosci 15(3): 367-370.

Bryant, R. A. (2001). "Posttraumatic stress disorder and traumatic brain injury: can they co-exist?" Clin Psychol Rev 21(6): 931-948.

Calder, A. J., A. D. Lawrence and A. W. Young (2001). "Neuropsychology of fear and loathing." Nat Rev Neurosci 2(5): 352-363.

Cawley, N., B. S. Solanky, N. Muhlert, C. Tur, R. A. Edden, C. A. Wheeler-Kingshott, D. H. Miller, A. J. Thompson and O. Ciccarelli (2015). "Reduced gamma-aminobutyric acid concentration is associated with physical disability in progressive multiple sclerosis." Brain 138(Pt 9): 2584-2595.

Chard, D. and S. A. Trip (2017). "Resolving the clinico-radiological paradox in multiple sclerosis." F1000Res 6: 1828.

Chaubard, J. L., C. Krishnamurthy, W. Yi, D. F. Smith and L. C. Hsieh-Wilson (2012). "Chemoenzymatic probes for detecting and imaging fucose-alpha(1-2)-galactose glycan biomarkers." J Am Chem Soc 134(10): 4489-4492.

Corrigan, J. D. and J. Bogner (2007). "Initial reliability and validity of the Ohio State University TBI Identification Method." J Head Trauma Rehabil 22(6): 318-329.

Cox, R. W. (1996). "AFNI: software for analysis and visualization of functional magnetic resonance neuroimages." Comput Biomed Res 29(3): 162-173.

Cross, K. J., K. T. Holmes, C. E. Mountford and P. E. Wright (1984). "Assignment of acyl chain resonances from membranes of mammalian cells by two-dimensional NMR methods." Biochemistry 23(25): 5895-5897.

Davidson, J. R., D. J. Stein, A. Y. Shalev and R. Yehuda (2004). "Posttraumatic stress disorder: acquisition, recognition, course, and treatment." J Neuropsychiatry Clin Neurosci 16(2): 135-147.

De Bellis, M. D., M. S. Keshavan, S. Spencer and J. Hall (2000). "N-Acetylaspartate concentration in the anterior cingulate of maltreated children and adolescents with PTSD." Am J Psychiatry 157(7): 1175-1177.

De Stefano, N., M. L. Stromillo, A. Giorgio, M. L. Bartolozzi, M. Battaglini, M. Baldini, E. Portaccio, M. P. Amato and M. P. Sormani (2016). "Establishing pathological cut-offs of brain atrophy rates in multiple sclerosis." J Neurol Neurosurg Psychiatry 87(1): 93-99.

Delikatny, E. J., W. E. Hull and C. E. Mountford (1991). "The effect of altering time domains and window functions in two-dimensional proton COSY spectra of biological specimens." Journal of Magnetic Resonance 94(3): 463-473.

Dua, V. K., B. N. Rao, S. S. Wu, V. E. Dube and C. A. Bush (1986). "Characterization of the oligosaccharide alditols from ovarian cyst mucin glycoproteins of blood group A using high pressure liquid chromatography (HPLC) and high field 1H NMR spectroscopy." Journal of Biological Chemistry 261(4): 1599-1608.

Dyck, B. A., M. G. Beyaert, M. A. Ferro and R. K. Mishra (2011). "Medial prefrontal cortical synapsin II knock-down induces behavioral abnormalities in the rat: examining synapsin II in the pathophysiology of schizophrenia." Schizophr Res 130(1-3): 250-259.

Eckart, C., J. Kaufmann, M. Kanowski, C. Tempelmann, H. Hinrichs, T. Elbert, H. J. Heinze and I. T. Kolassa (2012). "Magnetic resonance volumetry and spectroscopy of hippocampus and insula in relation to severe exposure of traumatic stress." Psychophysiology 49(2): 261-270.

Ernst, T., R. Kreis and B. D. Ross (1993). "Absolute quantitation of water and metabolites in the human brain. I. Compartiments and water." Journal of Magnetic Resonance B 102: 1-8.

Eshaghi, A., R. V. Marinescu, A. L. Young, N. C. Firth, F. Prados, M. Jorge Cardoso, C. Tur, F. De Angelis, N. Cawley, W. J. Brownlee, N. De Stefano, M. Laura Stromillo, M. Battaglini, S. Ruggieri, C. Gasperini, M. Filippi, M. A. Rocca, A. Rovira, J. Sastre-Garriga, J. J. G. Geurts, H. Vrenken, V. Wottschel, C. E. Leurs, B. Uitdehaag, L. Pirpamer, C. Enzinger, S. Ourselin, C. A. Gandini Wheeler-Kingshott, D. Chard, A. J. Thompson, F. Barkhof, D. C. Alexander and O. Ciccarelli (2018). "Progression of regional grey matter atrophy in multiple sclerosis." Brain 141(6): 1665-1677.

Ethofer, T., I. Mader, U. Seeger, G. Helms, M. Erb, W. Grodd, A. Ludolph and U. Klose (2003). "Comparison of longitudinal metabolite relaxation times in different regions of the human brain at 1.5 and 3 Tesla." Magn Reson Med 50(6): 1296-1301.

Etkin, A. and T. D. Wager (2007). "Functional neuroimaging of anxiety: a meta-analysis of emotional processing in PTSD, social anxiety disorder, and specific phobia." Am J Psychiatry 164(10): 1476-1488.

Evergren, E., F. Benfenati and O. Shupliakov (2007). "The synapsin cycle: a view from the synaptic endocytic zone." J Neurosci Res 85(12): 2648-2656.

Filippi, M. and M. A. Rocca (2011). "MR imaging of multiple sclerosis." Radiology 259(3): 659-681.

First, M. B., Spitzer, Robert L, Gibbon Miriam, and Williams, Janet B.W (2002). Structured Clinical Interview for DSM-IV-TR Axis I Disorders, Research Version, Patient Edition (SCID-I/P). New York, New York State Psychiatric Institute.

Fischl, B. (2012). "FreeSurfer." Neuroimage 62(2): 774-781.

Fleischer, V., R. Kolb, S. Groppa, F. Zipp, U. Klose and A. Groger (2016). "Metabolic Patterns in Chronic Multiple Sclerosis Lesions and Normal-appearing White Matter: Intraindividual Comparison by Using 2D MR Spectroscopic Imaging." Radiology 281(2): 536-543.

Foerster, B. R., M. Petrou, R. A. Edden, P. C. Sundgren, T. Schmidt-Wilcke, S. E. Lowe, S. E. Harte, D. J. Clauw and R. E. Harris (2012). "Reduced insular gamma-aminobutyric acid in fibromyalgia." Arthritis Rheum 64(2): 579-583.

Freeman, T., T. Kimbrell, L. Booe, M. Myers, D. Cardwell, D. M. Lindquist, J. Hart and R. A. Komoroski (2006). "Evidence of resilience: neuroimaging in former prisoners of war." Psychiatry Res 146(1): 59-64.

Freeman, T. W., D. Cardwell, C. N. Karson and R. A. Komoroski (1998). "In vivo proton magnetic resonance spectroscopy of the medial temporal lobes of subjects with combat-related posttraumatic stress disorder." Magn Reson Med 40(1): 66-71.

Freesurfer. (2015). "Freesurfer FAQ." from https://surfer.nmr.mgh.harvard.edu/fswiki/UserContributions/FAQ.

Gasparovic, C., T. Song, D. Devier, H. J. Bockholt, A. Caprihan, P. G. Mullins, S. Posse, R. E. Jung and L. A. Morrison (2006). "Use of tissue water as a concentration reference for proton spectroscopic imaging." Magn Reson Med 55(6): 1219-1226.

Geddes, J. W., K. Panchalingam, J. N. Keller and J. W. Pettegrew (1997). "Elevated phosphocholine and phosphatidylcholine following rat entorhinal cortex lesions." Neurobiol Aging 18(3): 305-308.

Gelineau-Morel, R., V. Tomassini, M. Jenkinson, H. Johansen-Berg, P. M. Matthews and J. Palace (2012). "The effect of hypointense white matter lesions on automated gray matter segmentation in multiple sclerosis." Hum Brain Mapp 33(12): 2802-2814.

Geurts, J. J., I. E. Reuling, H. Vrenken, B. M. Uitdehaag, C. H. Polman, J. A. Castelijns, F. Barkhof and P. J. Pouwels (2006). "MR spectroscopic evidence for thalamic and hippocampal, but not cortical, damage in multiple sclerosis." Magn Reson Med 55(3): 478-483.

Gilbertson, M. W., M. E. Shenton, A. Ciszewski, K. Kasai, N. B. Lasko, S. P. Orr and R. K. Pitman (2002). "Smaller hippocampal volume predicts pathologic vulnerability to psychological trauma." Nat Neurosci 5(11): 1242-1247.

Govindaraju, V., K. Young and A. A. Maudsley (2000). "Proton NMR chemical shifts and coupling constants for brain metabolites." NMR Biomed **13**(3): 129-153.

Grasso, G., C. Alafaci, M. Passalacqua, A. Morabito, M. Buemi, F. M. Salpietro and F. Tomasello (2002). "Assessment of human brain water content by cerebral bioelectrical impedance analysis: a new technique and its application to cerebral pathological conditions." Neurosurgery **50**(5): 1064-1072; discussion 1072-1064.

Greicius, M. D., G. Srivastava, A. L. Reiss and V. Menon (2004). "Default-mode network activity distinguishes Alzheimer's disease from healthy aging: evidence from functional MRI." Proc Natl Acad Sci U S A **101**(13): 4637-4642.

Guo, M., F. Chen, J. C. Guo, C. Z. Lu, X. L. Jiang, T. Liu, M. Li and W. Song (2012). "Study of the hippocampus and the anterior cingulate gyrus by proton MR spectroscopy in patients with post-traumatic stress disorder." Asian Pac J Trop Med **5**(2): 162-164.

Gussew, A., M. Erdtel, P. Hiepe, R. Rzanny and J. R. Reichenbach (2012). "Absolute quantitation of brain metabolites with respect to heterogeneous tissue compositions in (1)H-MR spectroscopic volumes." MAGMA **25**(5): 321-333.

Ham, B. J., J. Chey, S. J. Yoon, Y. Sung, D. U. Jeong, S. Ju Kim, M. E. Sim, N. Choi, I. G. Choi, P. F. Renshaw and I. K. Lyoo (2007). "Decreased N-acetyl-aspartate levels in anterior cingulate and hippocampus in subjects with post-traumatic stress disorder: a proton magnetic resonance spectroscopy study." Eur J Neurosci **25**(1): 324-329.

Harnett, N. G., K. H. Wood, E. W. Ference, 3rd, M. A. Reid, A. C. Lahti, A. J. Knight and D. C. Knight (2017). "Glutamate/glutamine concentrations in the dorsal anterior cingulate vary with Post-Traumatic Stress Disorder symptoms." J Psychiatr Res **91**: 169-176.

Harris, A. D., N. A. Puts and R. A. Edden (2015). "Tissue correction for GABA-edited MRS: Considerations of voxel composition, tissue segmentation, and tissue relaxations." J Magn Reson Imaging **42**(5): 1431-1440.

Harris, J. L., I. Y. Choi and W. M. Brooks (2015). "Probing astrocyte metabolism in vivo: proton magnetic resonance spectroscopy in the injured and aging brain." Front Aging Neurosci **7**: 202.

Hart, G. W. (2006). "Sweet insights into learning and memory." Nat Chem Biol **2**(2): 67-68.

Hartley, C. A., B. Fischl and E. A. Phelps (2011). "Brain structure correlates of individual differences in the acquisition and inhibition of conditioned fear." Cereb Cortex **21**(9): 1954-1962.

Healy, B. C., G. J. Buckle, E. N. Ali, S. Egorova, F. Khalid, S. Tauhid, B. I. Glanz, T. Chitnis, C. R. G. Guttmann, H. L. Weiner and R. Bakshi (2017). "Characterizing Clinical and MRI Dissociation in Patients with Multiple Sclerosis." J Neuroimaging **27**(5): 481-485.

Hilfiker, S., V. A. Pieribone, A. J. Czernik, H. T. Kao, G. J. Augustine and P. Greengard (1999). "Synapsins as regulators of neurotransmitter release." Philos Trans R Soc Lond B Biol Sci 354(1381): 269-279.

Hovatta, I., R. S. Tennant, R. Helton, R. A. Marr, O. Singer, J. M. Redwine, J. A. Ellison, E. E. Schadt, I. M. Verma, D. J. Lockhart and C. Barlow (2005). "Glyoxalase 1 and glutathione reductase 1 regulate anxiety in mice." Nature 438(7068): 662-666.

Howe, F. A., S. J. Barton, S. A. Cudlip, M. Stubbs, D. E. Saunders, M. Murphy, P. Wilkins, K. S. Opstad, V. L. Doyle, M. A. McLean, B. A. Bell and J. R. Griffiths (2003). "Metabolic profiles of human brain tumors using quantitative in vivo 1H magnetic resonance spectroscopy." Magn Reson Med 49(2): 223-232.

Hughes, K. C. and L. M. Shin (2011). "Functional neuroimaging studies of post-traumatic stress disorder." Expert Rev Neurother 11(2): 275-285.

Jansen, J. F., W. H. Backes, K. Nicolay and M. E. Kooi (2006). "1H MR spectroscopy of the brain: absolute quantification of metabolites." Radiology 240(2): 318-332.

Jenkinson, M., P. Bannister, M. Brady and S. Smith (2002). "Improved Optimization for the Robust and Accurate Linear Registration and Motion Correction of Brain Images." NeuroImage 17(2): 825-841.

Jenkinson, M. and S. Smith (2001). "A global optimisation method for robust affine registration of brain images." Med Image Anal 5(2): 143-156.

Jensen, J. E., S. C. Licata, D. Ongur, S. D. Friedman, A. P. Prescot, M. E. Henry and P. F. Renshaw (2009). "Quantification of J-resolved proton spectra in two-dimensions with LCModel using GAMMA-simulated basis sets at 4 Tesla." NMR Biomed 22(7): 762-769.

Kaiser, L. G., N. Schuff, N. Cashdollar and M. W. Weiner (2005). "Age-related glutamate and glutamine concentration changes in normal human brain: 1H MR spectroscopy study at 4 T." Neurobiol Aging 26(5): 665-672.

Kalovidouris SA, Gama CI, Lee LW and Hsieh-WilsonLC (2005). "A role for fucose R(1-2) galactose carbohydrates in neuronal growth." J. Am. Chem. Soc. 127: 1340–1341.

Kapeller, P., M. A. McLean, C. M. Griffin, D. Chard, G. J. Parker, G. J. Barker, A. J. Thompson and D. H. Miller (2001). "Preliminary evidence for neuronal damage in cortical grey matter and normal appearing white matter in short duration relapsing-remitting multiple sclerosis: a quantitative MR spectroscopic imaging study." J Neurol 248(2): 131-138.

Karl, A., M. Schaefer, L. S. Malta, D. Dorfel, N. Rohleder and A. Werner (2006). "A meta-analysis of structural brain abnormalities in PTSD." Neurosci Biobehav Rev 30(7): 1004-1031.

Karl, A. and A. Werner (2010). "The use of proton magnetic resonance spectroscopy in PTSD research--meta-analyses of findings and methodological review." Neurosci Biobehav Rev 34(1): 7-22.

Kasai, K., H. Yamasue, M. W. Gilbertson, M. E. Shenton, S. L. Rauch and R. K. Pitman (2008). "Evidence for acquired pregenual anterior cingulate gray matter loss from a twin study of combat-related posttraumatic stress disorder." Biol Psychiatry 63(6): 550-556.

Keeler, J. (2010). Understanding NMR spectroscopy. Chichester, U.K., John Wiley and Sons.

Kennedy, J. E., M. S. Jaffee, G. A. Leskin, J. W. Stokes, F. O. Leal and P. J. Fitzpatrick (2007). "Posttraumatic stress disorder and posttraumatic stress disorder-like symptoms and mild traumatic brain injury." J Rehabil Res Dev 44(7): 895-920.

Kessler, R. C., G. Andrews, L. J. Colpe, E. Hiripi, D. K. Mroczek, S. L. Normand, E. E. Walters and A. M. Zaslavsky (2002). "Short screening scales to monitor population prevalences and trends in non-specific psychological distress." Psychol Med 32(6): 959-976.

Khan, O., N. Seraji-Bozorgzad, F. Bao, S. Razmjou, C. Caon, C. Santiago, Z. Latif, R. Aronov, I. Zak, N. Ashtamker, S. Kolodny, C. Ford and Y. Sidi (2017). "The Relationship Between Brain MR Spectroscopy and Disability in Multiple Sclerosis: 20-Year Data from the U.S. Glatiramer Acetate Extension Study." J Neuroimaging 27(1): 97-106.

Kim, H. H., I. H. Jeong, J. S. Hyun, B. S. Kong, H. J. Kim and S. J. Park (2017). "Metabolomic profiling of CSF in multiple sclerosis and neuromyelitis optica spectrum disorder by nuclear magnetic resonance." PLoS One 12(7): e0181758.

Kimbrell, T., C. Leulf, D. Cardwell, R. A. Komoroski and T. W. Freeman (2005). "Relationship of in vivo medial temporal lobe magnetic resonance spectroscopy to documented combat exposure in veterans with chronic posttraumatic stress disorder." Psychiatry Res 140(1): 91-94.

King, N. S. (2008). "PTSD and traumatic brain injury: folklore and fact?" Brain Inj 22(1): 1-5.

Kitayama, N., S. Quinn and J. D. Bremner (2006). "Smaller volume of anterior cingulate cortex in abuse-related posttraumatic stress disorder." J Affect Disord 90(2-3): 171-174.

Kitayama, N., V. Vaccarino, M. Kutner, P. Weiss and J. D. Bremner (2005). "Magnetic resonance imaging (MRI) measurement of hippocampal volume in posttraumatic stress disorder: a meta-analysis." J Affect Disord 88(1): 79-86.

Kleene, R. and M. Schachner (2004). "Glycans and neural cell interactions." Nat Rev Neurosci 5(3): 195-208.

Klein, A., G. Lamblin, M. Lhermitte, P. Roussel, J. Breg, H. Van Halbeek and J. F. Vliegenthart (1988). "Primary structure of neutral oligosaccharides derived from respiratory-mucus glycoproteins of a patient suffering from bronchiectasis, determined by combination of 500-

MHz 1H-NMR spectroscopy and quantitative sugar analysis. 1. Structure of 16 oligosaccharides having the Gal beta(1----3)GalNAc-ol core (type 1) or the Gal beta(1----3)[GlcNAc beta(1----6)]GalNac-ol core (type 2)." Eur J Biochem 171(3): 631-642.

Klein, P., R. L. Somorjai and P. C. Lau (1988). "Distinctive properties of signal sequences from bacterial lipoproteins." Protein Eng 2(1): 15-20.

Koolman, J. and K.-H. Röhm (2013). Color atlas of biochemistry. Stuttgart, Thieme.

Kroenke, K., R. L. Spitzer and J. B. Williams (2002). "The PHQ-15: validity of a new measure for evaluating the severity of somatic symptoms." Psychosom Med 64(2): 258-266.

Krug M, Wagner M, Staak S and Smalla K H (1994). "Fucose and fucose-containing sugar epitopes enhance hippocampal longterm potentiation in the freely moving rat." Brain Res 643: 130–135.

Kurt Kroenke, R. L. S. (2002). "The PHQ-9: A New Depression Diagnostic and Severity Measure." Psychiatric Annals 32(9).

Kurtzke, J. F. (1983). "Rating neurologic impairment in multiple sclerosis: an expanded disability status scale (EDSS)." Neurology 33(11): 1444-1452.

Lanius, R. (2010). "Does neuroimaging research examining the pathophysiology of posttraumatic stress disorder require medication-free patients?" Journal of Psychiatry and Neuroscience 35(2): 80-89.

Lanius, R. and M. Olff (2017). "The neurobiology of PTSD." Eur J Psychotraumatol 8(1): 1314165.

Laule, C., I. M. Vavasour, G. R. Moore, J. Oger, D. K. Li, D. W. Paty and A. L. MacKay (2004). "Water content and myelin water fraction in multiple sclerosis. A T2 relaxation study." J Neurol 251(3): 284-293.

Lean, C. L., W. B. Mackinnon, E. J. Delikatny, R. H. Whitehead and C. E. Mountford (1992). "Cell-surface fucosylation and magnetic resonance spectroscopy characterization of human malignant colorectal cells." Biochemistry 31(45): 11095-11105.

Lean, C. L., W. B. Mackinnon and C. E. Mountford (1991). "Fucose in 1H COSY spectra of plasma membrane fragments shed from human malignant colorectal cells." Magn Reson Med 20(2): 306-311.

Lee, H., E. Caparelli, H. Li, A. Mandal, S. D. Smith, S. Zhang, T. V. Bilfinger and H. Benveniste (2013). "Computerized MRS voxel registration and partial volume effects in single voxel 1H-MRS." Magn Reson Imaging 31(7): 1197-1205.

Leech, R., S. Kamourieh, C. F. Beckmann and D. J. Sharp (2011). "Fractionating the default mode network: distinct contributions of the ventral and dorsal posterior cingulate cortex to cognitive control." J Neurosci 31(9): 3217-3224.

Leech, R. and D. J. Sharp (2014). "The role of the posterior cingulate cortex in cognition and disease." Brain 137(Pt 1): 12-32.

Lewerenz, J. and P. Maher (2015). "Chronic Glutamate Toxicity in Neurodegenerative Diseases-What is the Evidence?" Front Neurosci 9: 469.

Li, L., S. Chen, J. Liu, J. Zhang, Z. He and X. Lin (2006). "Magnetic resonance imaging and magnetic resonance spectroscopy study of deficits in hippocampal structure in fire victims with recent-onset posttraumatic stress disorder." Can J Psychiatry 51(7): 431-437.

Lim, M. K., C. H. Suh, H. J. Kim, S. T. Kim, J. S. Lee, M. H. Kang, J. H. Kim and J. H. Lee (2003). "Fire-related post-traumatic stress disorder: brain 1H-MR spectroscopic findings." Korean J Radiol 4(2): 79-84.

Lin, A. P., S. Ramadan, R. A. Stern, H. C. Box, C. J. Nowinski, B. D. Ross and C. E. Mountford (2015). "Changes in the neurochemistry of athletes with repetitive brain trauma: preliminary results using localized correlated spectroscopy." Alzheimers Res Ther 7(1): 13.

Listinsky, J. J., G. P. Siegal and C. M. Listinsky (2011). "The emerging importance of alpha-L-fucose in human breast cancer: a review." Am J Transl Res 3(4): 292-322.

Londono, A. C. and C. A. Mora (2014). "Nonconventional MRI biomarkers for in vivo monitoring of pathogenesis in multiple sclerosis." Neurol Neuroimmunol Neuroinflamm 1(4): e45.

Lorenzini CGA, Baldi E, Bucherelli C, Sacchetti B and Tassoni G (1997). "2-Deoxy-D-galactose effects on passive avoidance memorization in the rat." Neurobiol. Learn. Mem. 68: 317–324.

Luyten, P. R. and J. A. den Hollander (1986). "Observation of metabolites in the human brain by MR spectroscopy." Radiology 161(3): 795-798.

Maddock, R. J. and M. H. Buonocore (2012). "MR spectroscopic studies of the brain in psychiatric disorders." Curr Top Behav Neurosci 11: 199-251.

Maddock, R. J., A. S. Garrett and M. H. Buonocore (2003). "Posterior cingulate cortex activation by emotional words: fMRI evidence from a valence decision task." Hum Brain Mapp 18(1): 30-41.

Magarinos, A. M., B. S. McEwen, G. Flugge and E. Fuchs (1996). "Chronic psychosocial stress causes apical dendritic atrophy of hippocampal CA3 pyramidal neurons in subordinate tree shrews." J Neurosci 16(10): 3534-3540.

Mahmutyazicioglu, K., N. Konuk, H. Ozdemir, N. Atasoy, L. Atik and S. Gundogdu (2005). "Evaluation of the hippocampus and the anterior cingulate gyrus by proton MR spectroscopy in patients with post-traumatic stress disorder." Diagn Interv Radiol **11**(3): 125-129.

Mathews, V. P., P. B. Barker, S. J. Blackband, J. C. Chatham and R. N. Bryan (1995). "Cerebral metabolites in patients with acute and subacute strokes: concentrations determined by quantitative proton MR spectroscopy." AJR Am J Roentgenol **165**(3): 633-638.

MathWorks (1984-2014). MATLAB.

Matthies, H., S. Staak and M. Krug (1996). "Fucose and fucosyllactose enhance in-vitro hippocampal long-term potentiation." Brain Res **725**(2): 276-280.

Maudsley, A. A., C. Domenig, V. Govind, A. Darkazanli, C. Studholme, K. Arheart and C. Bloomer (2009). "Mapping of brain metabolite distributions by volumetric proton MR spectroscopic imaging (MRSI)." Magn Reson Med **61**(3): 548-559.

McEwen, B. S. (2000). "The neurobiology of stress: from serendipity to clinical relevance." Brain Res **886**(1-2): 172-189.

McEwen, B. S., J. Angulo, H. Cameron, H. M. Chao, D. Daniels, M. N. Gannon, E. Gould, S. Mendelson, R. Sakai, R. Spencer and et al. (1992). "Paradoxical effects of adrenal steroids on the brain: protection versus degeneration." Biol Psychiatry **31**(2): 177-199.

McFarlane, A., S. Hodson, M. Van Hooff and C. Davies (2010). Mental health in the Australian Defence Force: 2010 ADF Mental Health and Wellbeing Study: Full report. Canberra, Department of Defence.

McLean, M. A., F. G. Woermann, G. J. Barker and J. S. Duncan (2000). "Quantitative analysis of short echo time (1)H-MRSI of cerebral gray and white matter." Magn Reson Med **44**(3): 401-411.

Meloncelli, P. J., L. J. West and T. L. Lowary (2011). "Synthesis and NMR studies on the ABO histo-blood group antigens: synthesis of type III and IV structures and NMR characterization of type I-VI antigens." Carbohydr Res **346**(12): 1406-1426.

Mescher, M., H. Merkle, J. Kirsch, M. Garwood and R. Gruetter (1998). "Simultaneous in vivo spectral editing and water suppression." NMR Biomed **11**(6): 266-272.

Meyerhoff, D. J., A. Mon, T. Metzler and T. C. Neylan (2014). "Cortical gamma-aminobutyric acid and glutamate in posttraumatic stress disorder and their relationships to self-reported sleep quality." Sleep **37**(5): 893-900.

Michels, L., T. Schulte-Vels, M. Schick, R. L. O'Gorman, T. Zeffiro, G. Hasler and C. Mueller-Pfeiffer (2014). "Prefrontal GABA and glutathione imbalance in posttraumatic stress disorder: preliminary findings." Psychiatry Res **224**(3): 288-295.

Milad, M. R. and G. J. Quirk (2012). "Fear extinction as a model for translational neuroscience: ten years of progress." Annu Rev Psychol 63: 129-151.

Milad, M. R., G. J. Quirk, R. K. Pitman, S. P. Orr, B. Fischl and S. L. Rauch (2007). "A role for the human dorsal anterior cingulate cortex in fear expression." Biol Psychiatry 62(10): 1191-1194.

Miller, D. H., F. D. Lublin, M. P. Sormani, L. Kappos, O. Yaldizli, M. S. Freedman, B. A. C. Cree, H. L. Weiner, C. Lubetzki, H. P. Hartung, X. Montalban, B. M. J. Uitdehaag, D. G. MacManus, T. A. Yousry, C. A. M. Gandini Wheeler-Kingshott, B. Li, N. Putzki, M. Merschhemke, D. A. Haring and J. S. Wolinsky (2018). "Brain atrophy and disability worsening in primary progressive multiple sclerosis: insights from the INFORMS study." Ann Clin Transl Neurol 5(3): 346-356.

Moffett, J. R., B. Ross, P. Arun, C. N. Madhavarao and A. M. Namboodiri (2007). "N-Acetylaspartate in the CNS: from neurodiagnostics to neurobiology." Prog Neurobiol 81(2): 89-131.

Mohanakrishnan Menon, P., H. A. Nasrallah, J. A. Lyons, M. F. Scott and V. Liberto (2003). "Single-voxel proton MR spectroscopy of right versus left hippocampi in PTSD." Psychiatry Res 123(2): 101-108.

Mountford, C., S. Quadrelli, A. Lin and S. Ramadan (2015). "Six fucose-alpha(1-2) sugars and alpha-fucose assigned in the human brain using in vivo two-dimensional MRS." NMR Biomed 28(3): 291-296.

Mountford, C. E., S. Doran, C. L. Lean and P. Russell (2004). "Proton MRS can determine the pathology of human cancers with a high level of accuracy." Chemical Reviews 104(8): 3677-3704.

Mountford, C. E., P. Stanwell, A. Lin, S. Ramadan and B. Ross (2010). "Neurospectroscopy: the past, present and future." Chem Rev 110(5): 3060-3086.

Muhlert, N., M. Atzori, E. De Vita, D. L. Thomas, R. S. Samson, C. A. Wheeler-Kingshott, J. J. Geurts, D. H. Miller, A. J. Thompson and O. Ciccarelli (2014). "Memory in multiple sclerosis is linked to glutamate concentration in grey matter regions." J Neurol Neurosurg Psychiatry 85(8): 833-839.

Murray, M. E., S. A. Przybelski, T. G. Lesnick, A. M. Liesinger, A. Spychalla, B. Zhang, J. L. Gunter, J. E. Parisi, B. F. Boeve, D. S. Knopman, R. C. Petersen, C. R. Jack, Jr., D. W. Dickson and K. Kantarci (2014). "Early Alzheimer's disease neuropathology detected by proton MR spectroscopy." J Neurosci 34(49): 16247-16255.

Murrey HE, Gama CI, Kalovidouris SA, Luo WI, Driggers EM, Porton B and Hsieh-Wilson LC (2006). "Protein fucosylation regulates synapsin Ia/Ib expression and neuronal morphology in primary hippocampal neurons." Proc. Natl. Acad. Sci. U.S.A 103: 21–26.

Murrey, H. E., S. B. Ficarro, C. Krishnamurthy, S. E. Domino, E. C. Peters and L. C. Hsieh-Wilson (2009). "Identification of the plasticity-relevant fucose-alpha(1-2)-galactose proteome from the mouse olfactory bulb." Biochemistry 48(30): 7261-7270.

Murrey, H. E. and L. C. Hsieh-Wilson (2008). "The chemical neurobiology of carbohydrates." Chem Rev 108(5): 1708-1731.

Nacewicz, B. M., L. Angelos, K. M. Dalton, R. Fischer, M. J. Anderle, A. L. Alexander and R. J. Davidson (2012). "Reliable non-invasive measurement of human neurochemistry using proton spectroscopy with an anatomically defined amygdala-specific voxel." Neuroimage 59(3): 2548-2559.

Narayana, P. A. (2005). "Magnetic resonance spectroscopy in the monitoring of multiple sclerosis." J Neuroimaging 15(4 Suppl): 46S-57S.

Nardo, D., G. Hogberg, J. C. Looi, S. Larsson, T. Hallstrom and M. Pagani (2010). "Gray matter density in limbic and paralimbic cortices is associated with trauma load and EMDR outcome in PTSD patients." J Psychiatr Res 44(7): 477-485.

Neylan, T. C., N. Schuff, M. Lenoci, R. Yehuda, M. W. Weiner and C. R. Marmar (2003). "Cortisol levels are positively correlated with hippocampal N-acetylaspartate." Biol Psychiatry 54(10): 1118-1121.

O'Doherty, D. C., K. M. Chitty, S. Saddiqui, M. R. Bennett and J. Lagopoulos (2015). "A systematic review and meta-analysis of magnetic resonance imaging measurement of structural volumes in posttraumatic stress disorder." Psychiatry Res 232(1): 1-33.

Obert, D., G. Helms, M. B. Sattler, K. Jung, B. Kretzschmar, M. Bahr, P. Dechent, R. Diem and K. Hein (2016). "Brain Metabolite Changes in Patients with Relapsing-Remitting and Secondary Progressive Multiple Sclerosis: A Two-Year Follow-Up Study." PLoS One 11(9): e0162583.

Olney, J. W. (1986). Inciting Excitotoxic Cytocide Among Central Neurons. Excitatory Amino Acids and Epilepsy. Boston, MA, Springer. 203.

Oz, G., J. R. Alger, P. B. Barker, R. Bartha, A. Bizzi, C. Boesch, P. J. Bolan, K. M. Brindle, C. Cudalbu, A. Dincer, U. Dydak, U. E. Emir, J. Frahm, R. G. Gonzalez, S. Gruber, R. Gruetter, R. K. Gupta, A. Heerschap, A. Henning, H. P. Hetherington, F. A. Howe, P. S. Huppi, R. E. Hurd, K. Kantarci, D. W. Klomp, R. Kreis, M. J. Kruiskamp, M. O. Leach, A. P. Lin, P. R. Luijten, M. Marjanska, A. A. Maudsley, D. J. Meyerhoff, C. E. Mountford, S. J. Nelson, M. N. Pamir, J. W. Pan, A. C. Peet, H. Poptani, S. Posse, P. J. Pouwels, E. M. Ratai, B. D. Ross, T. W. Scheenen, C. Schuster, I. C. Smith, B. J. Soher, I. Tkac, D. B. Vigneron, R. A. Kauppinen and M. R. S. C. Group (2014). "Clinical proton MR spectroscopy in central nervous system disorders." Radiology 270(3): 658-679.

Pape, H. C. and D. Pare (2010). "Plastic synaptic networks of the amygdala for the acquisition, expression, and extinction of conditioned fear." Physiol Rev 90(2): 419-463.

Pennington, D. L., C. Abe, S. L. Batki and D. J. Meyerhoff (2014). "A preliminary examination of cortical neurotransmitter levels associated with heavy drinking in posttraumatic stress disorder." Psychiatry Res **224**(3): 281-287.

Phan, K. L., T. D. Wager, S. F. Taylor and I. Liberzon (2004). "Functional neuroimaging studies of human emotions." CNS Spectr **9**(4): 258-266.

Pohle W, Acosta L, Ruthrich H, Krug M and Matthies H (1987). "Incorporation of [H-3] fucose in rat hippocampal structures after conditioning by perforant path stimulation and after LTP-producing tetanization." Brain Res **410**: 245–256.

Provencher, S. (2015). "LC model Manual."

Provencher, S. W. (1993). "Estimation of metabolite concentrations from localized in vivo proton NMR spectra." Magn Reson Med **30**(6): 672-679.

Provencher, S. W. (2001). "Automatic quantitation of localized in vivo 1H spectra with LCModel." NMR Biomed **14**(4): 260-264.

Ramadan S, Andronesi O, Stanwell P, Lin A, Sorenson G and Mountford C (2011). "In Vivo Two Dimensional MR Spectroscopy Compares the Biochemistry of the Human Brain and Glioblastoma." Radiology **259**(2): 540-549.

Ramadan, S., O. C. Andronesi, P. Stanwell, A. P. Lin, A. G. Sorensen and C. E. Mountford (2011). "Use of in vivo two-dimensional MR spectroscopy to compare the biochemistry of the human brain to that of glioblastoma." Radiology **259**(2): 540-549.

Ramadan, S., J. Arm, J. Silcock, G. Santamaria, J. Buck, M. Roy, K. M. Leong, P. Lau, D. Clark, P. Malycha and C. Mountford (2015). "Lipid and Metabolite Deregulation in the Breast Tissue of Women Carrying BRCA1 and BRCA2 Genetic Mutations." Radiology **275**(3): 675-682.

Ramadan, S., A. Lin and P. Stanwell (2013). "Glutamate and glutamine: a review of in vivo MRS in the human brain." NMR Biomed **26**(12): 1630-1646.

Ramadan, S., E. M. Ratai, L. L. Wald and C. E. Mountford (2010). "In vivo 1D and 2D correlation MR spectroscopy of the soleus muscle at 7T." J Magn Reson **204**(1): 91-98.

Ranjith, G., R. Parvathy, V. Vikas, K. Chandrasekharan and S. Nair (2015). "Machine learning methods for the classification of gliomas: Initial results using features extracted from MR spectroscopy." Neuroradiol J **28**(2): 106-111.

Rao, N. P., G. Venkatasubramanian and B. N. Gangadhar (2011). "Proton magnetic resonance spectroscopy in depression." Indian J Psychiatry **53**(4): 307-311.

Revest, J. M., N. Kaouane, M. Mondin, A. Le Roux, F. Rouge-Pont, M. Vallee, J. Barik, F. Tronche, A. Desmedt and P. V. Piazza (2010). "The enhancement of stress-related memory by glucocorticoids depends on synapsin-Ia/Ib." Mol Psychiatry **15**(12): 1125, 1140-1151.

Richards, T. L. (1991). "Proton MR spectroscopy in multiple sclerosis: value in establishing diagnosis, monitoring progression, and evaluating therapy." AJR Am J Roentgenol **157**(5): 1073-1078.

Rohleder, N. and A. Karl (2006). "Role of endocrine and inflammatory alterations in comorbid somatic diseases of post-traumatic stress disorder." Minerva Endocrinol **31**(4): 273-288.

Roosendaal, S. D., K. Bendfeldt, H. Vrenken, C. H. Polman, S. Borgwardt, E. W. Radue, L. Kappos, D. Pelletier, S. L. Hauser, P. M. Matthews, F. Barkhof and J. J. Geurts (2011). "Grey matter volume in a large cohort of MS patients: relation to MRI parameters and disability." Mult Scler **17**(9): 1098-1106.

Ross JM, Öbergc J, Brenéd S and et al (2010). "High brain lactate is a hallmark of aging and caused by a shift in the lactate dehydrogenase A/B ratio." PNSA **104**(46): 20087-20092.

Rosso, I. M., D. J. Crowley, M. M. Silveri, S. L. Rauch and J. E. Jensen (2017). "Hippocampus Glutamate and N-Acetyl Aspartate Markers of Excitotoxic Neuronal Compromise in Posttraumatic Stress Disorder." Neuropsychopharmacology **42**(8): 1698-1705.

Rosso, I. M., M. R. Weiner, D. J. Crowley, M. M. Silveri, S. L. Rauch and J. E. Jensen (2014). "Insula and anterior cingulate GABA levels in posttraumatic stress disorder: preliminary findings using magnetic resonance spectroscopy." Depress Anxiety **31**(2): 115-123.

Rovira, A. and J. Alonso (2013). "1H magnetic resonance spectroscopy in multiple sclerosis and related disorders." Neuroimaging Clin N Am **23**(3): 459-474.

Rowland, B. C., H. Liao, F. Adan, L. Mariano, J. Irvine and A. P. Lin (2017). "Correcting for Frequency Drift in Clinical Brain MR Spectroscopy." J Neuroimaging **27**(1): 23-28.

Roxburgh, R. H., S. R. Seaman, T. Masterman, A. E. Hensiek, S. J. Sawcer, S. Vukusic, I. Achiti, C. Confavreux, M. Coustans, E. le Page, G. Edan, G. V. McDonnell, S. Hawkins, M. Trojano, M. Liguori, E. Cocco, M. G. Marrosu, F. Tesser, M. A. Leone, A. Weber, F. Zipp, B. Miterski, J. T. Epplen, A. Oturai, P. S. Sorensen, E. G. Celius, N. T. Lara, X. Montalban, P. Villoslada, A. M. Silva, M. Marta, I. Leite, B. Dubois, J. Rubio, H. Butzkueven, T. Kilpatrick, M. P. Mycko, K. W. Selmaj, M. E. Rio, M. Sa, G. Salemi, G. Savettieri, J. Hillert and D. A. Compston (2005). "Multiple Sclerosis Severity Score: using disability and disease duration to rate disease severity." Neurology **64**(7): 1144-1151.

Sapolsky, R. M. (1996). "Why stress is bad for your brain." Science **273**(5276): 749-750.

Saunders, D. E., F. A. Howe, A. van den Boogaart, J. R. Griffiths and M. M. Brown (1999). "Aging of the adult human brain: in vivo quantitation of metabolite content with proton magnetic resonance spectroscopy." J Magn Reson Imaging **9**(5): 711-716.

Schmidt, P., C. Gaser, M. Arsic, D. Buck, A. Forschler, A. Berthele, M. Hoshi, R. Ilg, V. J. Schmid, C. Zimmer, B. Hemmer and M. Muhlau (2012). "An automated tool for detection of FLAIR-hyperintense white-matter lesions in Multiple Sclerosis." Neuroimage **59**(4): 3774-3783.

Schuff, N., T. C. Neylan, S. Fox-Bosetti, M. Lenoci, K. W. Samuelson, C. Studholme, J. Kornak, C. R. Marmar and M. W. Weiner (2008). "Abnormal N-acetylaspartate in hippocampus and anterior cingulate in posttraumatic stress disorder." Psychiatry Res **162**(2): 147-157.

Schuff, N., T. C. Neylan, M. A. Lenoci, A. T. Du, D. S. Weiss, C. R. Marmar and M. W. Weiner (2001). "Decreased hippocampal N-acetylaspartate in the absence of atrophy in posttraumatic stress disorder." Biol Psychiatry **50**(12): 952-959.

Schulte, R. F. and P. Boesiger (2006). "ProFit: two-dimensional prior-knowledge fitting of J-resolved spectra." NMR Biomed **19**(2): 255-263.

Scioli-Salter, E. R., D. E. Forman, J. D. Otis, K. Gregor, I. Valovski and A. M. Rasmusson (2015). "The shared neuroanatomy and neurobiology of comorbid chronic pain and PTSD: therapeutic implications." Clin J Pain **31**(4): 363-374.

Seedat, S., J. S. Videen, C. M. Kennedy and M. B. Stein (2005). "Single voxel proton magnetic resonance spectroscopy in women with and without intimate partner violence-related posttraumatic stress disorder." Psychiatry Res **139**(3): 249-258.

Sehlmeyer, C., S. Schoning, P. Zwitserlood, B. Pfleiderer, T. Kircher, V. Arolt and C. Konrad (2009). "Human fear conditioning and extinction in neuroimaging: a systematic review." PLoS One **4**(6): e5865.

Shiee, N., P. L. Bazin, K. M. Zackowski, S. K. Farrell, D. M. Harrison, S. D. Newsome, J. N. Ratchford, B. S. Caffo, P. A. Calabresi, D. L. Pham and D. S. Reich (2012). "Revisiting brain atrophy and its relationship to disability in multiple sclerosis." PLoS One **7**(5): e37049.

Shin, L. M., N. B. Lasko, M. L. Macklin, R. D. Karpf, M. R. Milad, S. P. Orr, J. M. Goetz, A. J. Fischman, S. L. Rauch and R. K. Pitman (2009). "Resting metabolic activity in the cingulate cortex and vulnerability to posttraumatic stress disorder." Arch Gen Psychiatry **66**(10): 1099-1107.

Shu, X.-J., L. Xue, W. Liu, F.-Y. Chen, C. Zhu, X.-H. Sun, X.-P. Wang, Z.-C. Liu and H. Zhao (2013). More vulnerability of left than right hippocampal damage in right-handed patients with post-traumatic stress disorder. Psychiatry research. **212:** 237-244.

Sierra-Mercado, D., N. Padilla-Coreano and G. J. Quirk (2011). "Dissociable roles of prelimbic and infralimbic cortices, ventral hippocampus, and basolateral amygdala in the expression and extinction of conditioned fear." Neuropsychopharmacology **36**(2): 529-538.

Siqveland, J., T. Ruud and E. Hauff (2017). "Post-traumatic stress disorder moderates the relationship between trauma exposure and chronic pain." Eur J Psychotraumatol **8**(1): 1375337.

Smith, M. E. (2005). "Bilateral hippocampal volume reduction in adults with post-traumatic stress disorder: a meta-analysis of structural MRI studies." Hippocampus **15**(6): 798-807.

Smith, S. M. (2002). "Fast robust automated brain extraction." Hum Brain Mapp 17(3): 143-155.

Smith, S. M., M. Jenkinson, M. W. Woolrich, C. F. Beckmann, T. E. Behrens, H. Johansen-Berg, P. R. Bannister, M. De Luca, I. Drobnjak, D. E. Flitney, R. K. Niazy, J. Saunders, J. Vickers, Y. Zhang, N. De Stefano, J. M. Brady and P. M. Matthews (2004). "Advances in functional and structural MR image analysis and implementation as FSL." Neuroimage 23 Suppl 1: S208-219.

Smith, S. M., Y. Zhang, M. Jenkinson, J. Chen, P. M. Matthews, A. Federico and N. De Stefano (2002). "Accurate, robust, and automated longitudinal and cross-sectional brain change analysis." Neuroimage 17(1): 479-489.

Soreni, N., M. D. Noseworthy, T. Cormier, W. K. Oakden, S. Bells and R. Schachar (2006). "Intraindividual variability of striatal (1)H-MRS brain metabolite measurements at 3 T." Magn Reson Imaging 24(2): 187-194.

Sormani, M. P., L. Kappos, E. W. Radue, J. Cohen, F. Barkhof, T. Sprenger, D. Piani Meier, D. Haring, D. Tomic and N. De Stefano (2017). "Defining brain volume cutoffs to identify clinically relevant atrophy in RRMS." Mult Scler 23(5): 656-664.

Spitzer, R. L., K. Kroenke, J. B. Williams and B. Lowe (2006). "A brief measure for assessing generalized anxiety disorder: the GAD-7." Arch Intern Med 166(10): 1092-1097.

Stanwell P, Siddall P, Keshava N, Cocuzzo D, Ramadan S, Lin A, Herbert D, Craig A, Tran Y, Middleton J, Gautam S, Cousins M and M. C. (2010). "Neuro magnetic resonance spectroscopy using wavelet decomposition and statistical testing identifies biochemical changes in people with spinal cord injury and pain." NeuroImage 53.(2): 544-552.

Steen, C., M. D'Haeseleer, J. M. Hoogduin, Y. Fierens, M. Cambron, J. P. Mostert, D. J. Heersema, M. W. Koch and J. De Keyser (2013). "Cerebral white matter blood flow and energy metabolism in multiple sclerosis." Mult Scler 19(10): 1282-1289.

Strecker G, Wieruszeski JM, Michalski JC and M. J. (1992). "1H-and 13C-n.m.r. spectroscopy of 2-oxo-3-deoxy-D-glycero-D- galactononulosonic acid-containing oligosaccharide-alditols bearing Lewis X, Lewis Y and A-Lewis Y determinants isolated from the jelly coat of Pleurodeles waltl egg." Biochem J 1(287): 905-909.

Sunanda, M. S. Rao and T. R. Raju (1995). "Effect of chronic restraint stress on dendritic spines and excrescences of hippocampal CA3 pyramidal neurons--a quantitative study." Brain Res 694(1-2): 312-317.

Tallan, H. H. (1957). "Studies on the distribution of N-acetyl-L-aspartic acid in brain." J Biol Chem 224(1): 41-45.

Tallan, H. H., S. Moore and W. H. Stein (1956). "N-Acetyl-L-aspartic acid in brain." J Biol Chem 219(1): 257-264.

Tan, M. L., B. A. Dyck, J. Gabriele, R. P. Daya, N. Thomas, C. Sookram, D. Basu, M. A. Ferro, V. Z. Chong and R. K. Mishra (2014). "Synapsin II gene expression in the dorsolateral prefrontal cortex of brain specimens from patients with schizophrenia and bipolar disorder: effect of lifetime intake of antipsychotic drugs." Pharmacogenomics J **14**(1): 63-69.

Terpstra, M., I. Cheong, T. Lyu, D. K. Deelchand, U. E. Emir, P. Bednarik, L. E. Eberly and G. Oz (2016). "Test-retest reproducibility of neurochemical profiles with short-echo, single-voxel MR spectroscopy at 3T and 7T." Magn Reson Med **76**(4): 1083-1091.

Thomas, M. A., N. Hattori, M. Umeda, T. Sawada and S. Naruse (2003). "Evaluation of two-dimensional L-COSY and JPRESS using a 3 T MRI scanner: from phantoms to human brain in vivo." NMR Biomed **16**(5): 245-251.

Thomas, M. A., K. Yue, N. Binesh, P. Davanzo, A. Kumar, B. Siegel, M. Frye, J. Curran, R. Lufkin, P. Martin and B. Guze (2001). "Localized two-dimensional shift correlated MR spectroscopy of human brain." Magn Reson Med **46**(1): 58-67.

Tisell, A., O. D. Leinhard, J. B. Warntjes, A. Aalto, O. Smedby, A. M. Landtblom and P. Lundberg (2013). "Increased concentrations of glutamate and glutamine in normal-appearing white matter of patients with multiple sclerosis and normal MR imaging brain scans." PLoS One **8**(4): e61817.

Tovote, P., J. P. Fadok and A. Luthi (2015). "Neuronal circuits for fear and anxiety." Nat Rev Neurosci **16**(6): 317-331.

Traboulsee, A., J. H. Simon, L. Stone, E. Fisher, D. E. Jones, A. Malhotra, S. D. Newsome, J. Oh, D. S. Reich, N. Richert, K. Rammohan, O. Khan, E. W. Radue, C. Ford, J. Halper and D. Li (2016). "Revised Recommendations of the Consortium of MS Centers Task Force for a Standardized MRI Protocol and Clinical Guidelines for the Diagnosis and Follow-Up of Multiple Sclerosis." AJNR Am J Neuroradiol **37**(3): 394-401.

Tu, Z., Y. N. Lin and C. H. Lin (2013). "Development of fucosyltransferase and fucosidase inhibitors." Chem Soc Rev **42**(10): 4459-4475.

Van Halbeek, H., J. Breg, J. F. Vliegenthart, A. Klein, G. Lamblin and P. Roussel (1988). "Isolation and structural characterization of low-molecular-mass monosialyl oligosaccharides derived from respiratory-mucus glycoproteins of a patient suffering from bronchiectasis." Eur J Biochem **177**(2): 443-460.

Vidal-Gonzalez, I., B. Vidal-Gonzalez, S. L. Rauch and G. J. Quirk (2006). "Microstimulation reveals opposing influences of prelimbic and infralimbic cortex on the expression of conditioned fear." Learn Mem **13**(6): 728-733.

Villarreal, G., H. Petropoulos, D. A. Hamilton, L. M. Rowland, W. P. Horan, J. A. Griego, M. Moreshead, B. L. Hart and W. M. Brooks (2002). "Proton magnetic resonance spectroscopy of the hippocampus and occipital white matter in PTSD: preliminary results." Can J Psychiatry **47**(7): 666-670.

Von Korff, M., J. Ormel, F. J. Keefe and S. F. Dworkin (1992). "Grading the severity of chronic pain." Pain 50(2): 133-149.

Wang, Y. and S. J. Li (1998). "Differentiation of metabolic concentrations between gray matter and white matter of human brain by in vivo 1H magnetic resonance spectroscopy." Magn Reson Med 39(1): 28-33.

Wansapura, J. P., S. K. Holland, R. S. Dunn and W. S. Ball, Jr. (1999). "NMR relaxation times in the human brain at 3.0 tesla." J Magn Reson Imaging 9(4): 531-538.

Weathers, F. W., Blake, D.D., Schnurr, P.P., Kaloupek, D.G., Marx, B.P., & Keane, T.M. (2013). The Life Events Checklist for DSM-5 (LEC-5).

Weathers, F. W., B. T. Litz, D. S. Herman, J. A. Huska and T. M. Keane (1993). The PTSD Checklist (PCL): Reliablity, validity, and diagnostic utility. 9th Annual Conference of the ISTSS. San Antonio.

Weber-Fahr, W., G. Ende, D. F. Braus, P. Bachert, B. J. Soher, F. A. Henn and C. Buchel (2002). "A fully automated method for tissue segmentation and CSF-correction of proton MRSI metabolites corroborates abnormal hippocampal NAA in schizophrenia." Neuroimage 16(1): 49-60.

Wegner, C., M. M. Esiri, S. A. Chance, J. Palace and P. M. Matthews (2006). "Neocortical neuronal, synaptic, and glial loss in multiple sclerosis." Neurology 67(6): 960-967.

Williams, P. G., J. K. Saunders, M. Dyne, C. E. Mountford and K. T. Holmes (1988). "Application of a T2-filtered COSY experiment to identify the origin of slowly relaxing species in normal and malignant tissue." Magn Reson Med 7(4): 463-471.

Wolf, R. C., M. S. Pujara, J. C. Motzkin, J. P. Newman, K. A. Kiehl, J. Decety, D. S. Kosson and M. Koenigs (2015). "Interpersonal traits of psychopathy linked to reduced integrity of the uncinate fasciculus." Hum Brain Mapp 36(10): 4202-4209.

Woodward, S. H., D. G. Kaloupek, C. C. Streeter, C. Martinez, M. Schaer and S. Eliez (2006). "Decreased anterior cingulate volume in combat-related PTSD." Biol Psychiatry 59(7): 582-587.

Wright, L. C., G. L. May, P. Gregory, M. Dyne, K. T. Holmes, P. G. Williams and C. E. Mountford (1988). "Inhibition of metastatic potential by fucosidase: an NMR study identifies a cell surface metastasis marker." J Cell Biochem 37(1): 49-59.

Yang, Z. Y., H. Quan, Z. L. Peng, Y. Zhong, Z. J. Tan and Q. Y. Gong (2015). "Proton magnetic resonance spectroscopy revealed differences in the glutamate + glutamine/creatine ratio of the anterior cingulate cortex between healthy and pediatric post-traumatic stress disorder patients diagnosed after 2008 Wenchuan earthquake." Psychiatry Clin Neurosci 69(12): 782-790.

Yetkin, M. F., M. Mirza and H. Donmez (2016). "Monitoring interferon beta treatment response with magnetic resonance spectroscopy in relapsing remitting multiple sclerosis." Medicine (Baltimore) **95**(36): e4782.

Zhang, Y., M. Brady and S. Smith (2001). "Segmentation of brain MR images through a hidden Markov random field model and the expectation-maximization algorithm." IEEE Trans Med Imaging **20**(1): 45-57.

Appendix

List of publications

Journal Articles

Mountford, C., **S. Quadrelli**, A. Lin and S. Ramadan (2015). "Six fucose-alpha(1-2) sugars and alpha-fucose assigned in the human brain using in vivo two-dimensional MRS." Nmr in Biomedicine **28**(3): 291-296.

Quadrelli, S., C. Mountford and S. Ramadan (2016). "Hitchhiker's Guide to Voxel Segmentation for Partial Volume Correction of In Vivo Magnetic Resonance Spectroscopy." Magnetic Resonance Insights **9**: 1-8.

Arm, J., O. Al-iedani, **S. Quadrelli**, K. Ribbons, R. Lea, J. Lechner-Scott and S. Ramadan (2018). "Test-retest repeatability of neurometabolites with two dimensional localised correlation spectroscopy (2D L-COSY) at 3Tesla." Journal of Magnetic Resonance Imaging.

Quadrelli, S., C. Mountford and S. Ramadan (2018). "Systematic review of *in-vivo* neuro Magnetic Resonance Spectroscopy for the assessment of Posttraumatic Stress Disorder" Psychiatric Research: Neuroimaging.

Under Review

Quadrelli, S., N. Tosh, A. Urquhart, K. Tricky, R. Tremewan, G. Galloway, L. Rich, R. Lea, P. Malycha, and C. Mountford. "Posttraumatic Stress Disorder Affects Fucose-α(1–2)-glycans in the Human Brain: Preliminary Findings of Neuro Deregulation using In Vivo Two Dimensional Neuro MR Spectroscopy" Translational Psychiatry. Submitted 15/5/18.

Gholizadeh, N., Pundavela, J., Dona, A., **Quadrelli, S.**, Biswas, T. and Ramadan, S. "Magnetic Resonance Spectroscopy: Potential Role in Clinical Management In Prostate Cancer" Journal of Biological Sciences. Submitted 16/2/18.

Submitted

Quadrelli, S., K. Ribbons, J.Arm, O. Al-iedani, , J. Lechner-Scott, R. Lea and S. Ramadan (2018). "2D *in-vivo* L-COSY spectroscopy identifies neurometabolite alterations in multiple sclerosis" Radiology.

In preparation

Quadrelli, S., N. Tosh, A. Urquhart, K. Tricky, R. Tremewan, G. Galloway, L. Rich, R. Lea, P. Malycha, and C. Mountford. “Structural connectivity of Postraumatic stress disorder: a diffusion MRI connectometry study”.

Tosh, N., S. Quadrelli, T. Gasss, G. Galloway, and C. Mountford. “Two Additional Fucose-α(1–2)-Glycans, Known To Be Involved In Memory, Learning And Neuronal Health, Assigned In The Human Brain Taking The Total To Seven.”

Conference Abstracts

Quadrelli, S. G., S. Ramadan, A. Lin, J. Dimitrikov and C. E. Mountford (2014). α-Fucose increased in the brain of chronic pelvic pain syndrome patients with inflammation at onset recorded by 2D L-COSY. ISMRM 2014 Annual Scientific Meeting Proceedings: 3746.

Buck, J., S. Ramadan, L. Best, J. Silcock, J. Arm, S. Quadrelli, G. Santamaria, K. M. Leong, P. Lau, P. Malycha and others (2015). "Alterations to breast tissue chemistry in women at risk of cancer: 2D MR spectroscopy in vivo study." ISMRM 2015 Annual Scientific Meeting Proceedings.

Ribbons, K., S. Quadrelli, J. Lechner-Scott, O. Al-Iedani, J. Arm, C. E. Mountford and S. Ramadan (2015). "2D MR spectroscopy can identify molecules differentiating MS from healthy controls." ECTRIMS 2015 Annual Scientific Meeting Proceedings.

Santamaria, G., J. Buck, L. Best, D. Clark, J. Silcock, P. Lau, S. Ramadan, S. Quadrelli, P. Malycha and C. E. Mountford (2016). "Breast tissue lipid and metabolite deregulation precedes malignant transformation in women with BRCA gene mutations: A longitudinal study." ISMRM 2016 Annual Scientific Meeting Proceedings.

Quadrelli, S. G., G. Holtmann, N. Talley, S. Ramadan and C. E. Mountford (2016). "Neurochemical alterations detected in Irritable Bowel Syndrome using 2D L-COSY." ISMRM 2016 Annual Scientific Meeting Proceedings.

Galloway, G. J., S. G. Quadrelli, A. J. Urquhart, K. Trickey, P. Malycha, T. Keane and C. E. Mountford (2017). "Neuro 2D correlated spectroscopy identifies neuro deregulation in soldiers exposed to blast prior to discernible changes By conventional imaging." ISMRM 2017 Annual Scientific Meeting Proceedings.

Questionnaire developed for PTSD study

MRS Questionnaire - Post Traumatic Stress Disorder (ver 4)

The purpose of this study is to characterise the brain chemistry of patients who have been diagnosed with Post Traumatic Stress Disorder (PTSD) using Magnetic Resonance Spectroscopy (MRS). The brain chemistry of participants with PTSD will be compared to controls or patients who haven't been diagnosed with PTSD.

This is a research project being conducted by Prof Carolyn Mountford from the Centre for Magnetic Resonance in Health at the University of Newcastle. The survey questions will be about your previous medical history, alcohol, illicit drug use, depression, anxiety and previous traumatic events. The procedure involves filling an online survey that will take approximately 30 minutes, depending on how you answer the questions.

Your responses will be confidential and we do not collect identifying information such as your name, email address or IP address. All data will be transfered using a secure SLL internet connection.

Your participation in this research study is voluntary. You may choose not to participate. If you decide to participate in this research survey, you may withdraw at any time. If you decide not to participate in this study or if you withdraw from participating at any time you will not be penalised.

We will do our best to keep your information confidential. All data is stored in a password protected electronic format. To help protect your confidentiality, the surveys do not contain information that will personally identify you. The results of this study will be used for scholarly purposes only and may be shared with University of Newcastle and data analysis specialists. If you are participating in this study as a control your data will be stored for fifteen years and may be used as control data in future projects undertaken by the Centre for Magnetic Resonance in medicine at the University of Newcastle, as explained in the participant information sheet.

If you have any questions about the research study, please contact Dr Scott Quadrelli - scott.quadrelli@newcastle.edu.au.

If you find involvment in the survey distressing in any way you can talk to someone about it:

- **Lifeline** 131 114
- **Your General Practitioner**

All research in Australia involving humans is reviewed by an independent group of people called a Human Research Ethics Committee (HREC). The ethical aspects of this research project have been approved by the HREC of Hunter New England Human Research Ethics Committee of Hunter New England Local Health District, Reference [13/04/17/4.04].

ELECTRONIC CONSENT: Please select your choice below.

Clicking on the "Agree" button below indicates that:

- you have read the above information
- you voluntarily agree to participate
- you are at least 18 years of age
- you agree for you data to be stored for 5 years and if suitable to be used in other research projects

If you do not wish to participate in the research study, please decline participation by clicking on the "disagree" button.

There are 81 questions in this survey

Demographics

Participant Demographics

Please enter your Questionnaire ID *

Please write your answer here:

You would have been supplied this by email.

Please enter you date of birth: *

Please enter a date:

Gender: *

Please choose **only one** of the following:

- ○ Female
- ○ Male

Have you had any inflammation or infections recently ? *

Please choose **only one** of the following:

- ○ In the last 1-7 days
- ○ In the last 8 -21 days
- ○ In the last year
- ○ No recent inflammation or infections

Make a comment on your choice here:

Please comment on where you have experienced inflammation or infections.

What is your highest level of education ? *

Please choose **only one** of the following:

- ○ Primary school
- ○ Secondary school up to grade 10
- ○ Secondary school grades 11-12
- ○ Certificate (trade, apprenticeship, technicians etc)
- ○ Diploma (associate, undergraduate)
- ○ Bachelor degree
- ○ Post-graduate qualification

Previous Medical History

We would like to know whether a medical doctor has diagnosed you with, or treated you for, any of the following medical problems or conditions. If YES, please indicate the year you were first diagnosed, whether you have been treated by a medical doctor for this condition in the past year, and whether you have taken any medications for the condition in the past month.

This could include medications requiring a prescription or other medications bought 'over the counter' such as: Ventolin, Aspirin, and Voltaren.

Has a medical doctor has diagnosed you with, or treated you for, any of the following medical problems or conditions?

	Yes	Treated by a doctor in the past year?	Medication taken in the past month?
Hypertension	☐	☐	☐
Heart attack / Myocardial infarction	☐	☐	☐
Angina	☐	☐	☐
High cholesterol	☐	☐	☐
Stroke	☐	☐	☐
Heart failure / Cardiac failure	☐	☐	☐
Epilepsy	☐	☐	☐
Migraines	☐	☐	☐
Motor neurone disease	☐	☐	☐
Multiple sclerosis	☐	☐	☐
Pneumonia	☐	☐	☐
Stomach or duodenal ulcers	☐	☐	☐
Colitis / Crohn's disease	☐	☐	☐
Functional dyspepsia	☐	☐	☐
Hepatitis	☐	☐	☐
Cirrhosis of the liver	☐	☐	☐
Polyp/s in the bowel	☐	☐	☐
Kidney disease e.g. stones, infection, bleeding	☐	☐	☐
Bladder disease e.g. infection, bleeding	☐	☐	☐
Diabetes	☐	☐	☐
Temporomandibular Joint (TMJ) Dysfunction	☐	☐	☐
Traumatic Brain Injury	☐	☐	☐
Fibrositis or fibromyalgia	☐	☐	☐
Eye or vision problems e.g. glaucoma	☐	☐	☐
Sinus problems	☐	☐	☐
Hearing loss	☐	☐	☐
Dermatitis	☐	☐	☐
Eczema	☐	☐	☐
Malignant melanoma	☐	☐	☐
Other skin cancer e.g. squamous cell or basal cell skin cancers	☐	☐	☐
Chronic Fatigue Syndrome	☐	☐	☐
Impotence	☐	☐	☐
Alcohol abuse or dependency	☐	☐	☐
Drug abuse or dependency	☐	☐	☐
Anxiety or stress	☐	☐	☐
Depression	☐	☐	☐
Post Traumatic Stress Disorder	☐	☐	☐

{PMHX_SQ1_1.question}

Have you been diagnosed with any cancer, tumour or malignancy, not listed above? *

Please choose **only one** of the following:

- ○ Yes
- ○ No

Have you been diagnosed with any other psychiatric or psychological condition needing treatment or counselling not listed above? *

Please choose **only one** of the following:

- ○ Yes
- ○ No

Please indicate what diagnosis was made:

Only answer this question if the following conditions are met:
Answer was 'Yes' at question '9 [PMHX_4]' (Have you been diagnosed with any other psychiatric or psychological condition needing treatment or counselling not listed above?)

Please write your answer here:

Please list any other medical problems or conditions which a medical doctor has diagnosed you with, or treated you for?

Please write your answer(s) here:

Medical Condition

What year were you diagnosed by medical doctor?

Have you been treated by a doctor is the last year for this condition?

Have you taken medications in the last month for this condition?

Please list any other medical problems or conditions which a medical doctor has diagnosed you with, or treated you for?

Only answer this question if the following conditions are met:
Answer was NOT at question '11 [PMHX_5]' (Please list any other medical problems or conditions which a medical doctor has diagnosed you with, or treated you for?)

Please write your answer(s) here:

Medical Condition

What year were you diagnosed by medical doctor?

Have you been treated by a doctor is the last year for this condition?

Have you taken medications in the last month for this condition?

Previous Health (TBI)

We would now like to ask you about injuries to your head or neck that you may have had at anytime in your life.

In your lifetime, have you ever been hospitalized or treated in an emergency room following an injury to your head or neck ? *

Please choose **only one** of the following:

- ○ Yes
- ○ No

Try to also think about any childhood injuries you remember or were told about.

What was the cause of the injury to your head or neck ?

Only answer this question if the following conditions are met:
Answer was 'Yes' at question '13 [TBI1]' (In your lifetime, have you ever been hospitalized or treated in an emergency room following an injury to your head or neck ?)

Please write your answer here:

Please record only one response.

How long were you knocked out or did you lose conciousness (LOC) for? *

Only answer this question if the following conditions are met:
Answer was 'Yes' at question '13 [TBI1]' (In your lifetime, have you ever been hospitalized or treated in an emergency room following an injury to your head or neck ?)

Please choose **only one** of the following:

- ○ No Loss of Conciousness (LOC)
- ○ Less than 30 minutes
- ○ 30 minutes to 24 hours
- ○ Greater than 24 hours

Were you dazed or did you have a gap in your memory from the injury? *

Only answer this question if the following conditions are met:
Answer was 'No Loss of Conciousness (LOC)' at question '15 [TBILOC]' (How long were you knocked out or did you lose conciousness (LOC) for?)

Please choose **only one** of the following:

- ○ Yes
- ○ No

What age were you when this injury occured ?

Only answer this question if the following conditions are met:
Answer was 'Yes' at question '13 [TBI1]' (In your lifetime, have you ever been hospitalized or treated in an emergency room following an injury to your head or neck ?)

Only numbers may be entered in this field.

Please write your answer here:

In your lifetime, have you ever injured your head or neck in a car accident or from crashing some other moving vehicle like a bicycle, motorcycle or ATV? *

Please choose **only one** of the following:

- ○ Yes
- ○ No

What was the cause of the injury to your head or neck ?

Only answer this question if the following conditions are met:
Answer was 'Yes' at question '18 [TBI2]' (In your lifetime, have you ever injured your head or neck in a car accident or from crashing some other moving vehicle like a bicycle, motorcycle or ATV?)

Please write your answer here:

Please record only one response.

How long were you knocked out or did you lose conciousness (LOC) for? *

Only answer this question if the following conditions are met:
Answer was 'Yes' at question '18 [TBI2]' (In your lifetime, have you ever injured your head or neck in a car accident or from crashing some other moving vehicle like a bicycle, motorcycle or ATV?)

Please choose **only one** of the following:

- ○ No Loss of Conciousness (LOC)
- ○ Less than 30 minutes
- ○ 30 minutes to 24 hours
- ○ Greater than 24 hours

Were you dazed or did you have a gap in your memory from the injury? *

Only answer this question if the following conditions are met:
Answer was 'No Loss of Conciousness (LOC)' at question '20 [TBILOC1]' (How long were you knocked out or did you lose conciousness (LOC) for?)

Please choose **only one** of the following:

- ○ Yes
- ○ No

What age were you when this injury occured ?

Only answer this question if the following conditions are met:
Answer was 'Yes' at question '18 [TBI2]' (In your lifetime, have you ever injured your head or neck in a car accident or from crashing some other moving vehicle like a bicycle, motorcycle or ATV?)

Only numbers may be entered in this field.

Please write your answer here:

In your lifetime, have you ever injured your head or neck in a fall or from being hit by something (for example, falling from a bike or horse, rollerblading, falling on ice, being hit by a rock)? Have you ever injured your head or neck playing sports or on the playground? *

Please choose **only one** of the following:

- ○ Yes
- ○ No

What was the cause of the injury to your head or neck ?

Only answer this question if the following conditions are met:
Answer was 'Yes' at question '23 [TBI3]' (In your lifetime, have you ever injured your head or neck in a fall or from being hit by something (for example, falling from a bike or horse, rollerblading, falling on ice, being hit by a rock)? Have you ever injured your head or neck playing sports or on the playground?)

Please write your answer here:

Please record only one response.

How long were you knocked out or did you lose conciousness (LOC) for? *

Only answer this question if the following conditions are met:
Answer was 'Yes' at question '23 [TBI3]' (In your lifetime, have you ever injured your head or neck in a fall or from being hit by something (for example, falling from a bike or horse, rollerblading, falling on ice, being hit by a rock)? Have you ever injured your head or neck playing sports or on the playground?)

Please choose **only one** of the following:

- ○ No Loss of Conciousness (LOC)
- ○ Less than 30 minutes
- ○ 30 minutes to 24 hours
- ○ Greater than 24 hours

Were you dazed or did you have a gap in your memory from the injury? *

Only answer this question if the following conditions are met:
Answer was 'No Loss of Conciousness (LOC)' at question '25 [TBILOC2]' (How long were you knocked out or did you lose conciousness (LOC) for?)

Please choose **only one** of the following:

- ○ Yes
- ○ No

What age were you when this injury occured ?

Only answer this question if the following conditions are met:
Answer was 'Yes' at question '23 [TBI3]' (In your lifetime, have you ever injured your head or neck in a fall or from being hit by something (for example, falling from a bike or horse, rollerblading, falling on ice, being hit by a rock)? Have you ever injured your head or neck playing sports or on the playground?)

Only numbers may be entered in this field.

Please write your answer here:

In your lifetime, have you ever injured your head or neck in a fight, from being hit by someone, or from being shaken violently? Have you ever been shot in the head? *

Please choose **only one** of the following:

- ○ Yes
- ○ No

What was the cause of the injury to your head or neck ?

Only answer this question if the following conditions are met:
Answer was 'Yes' at question '28 [TBI4]' (In your lifetime, have you ever injured your head or neck in a fight, from being hit by someone, or from being shaken violently? Have you ever been shot in the head?)

Please write your answer here:

Please record only one response.

How long were you knocked out or did you lose conciousness (LOC) for? *

Only answer this question if the following conditions are met:
Answer was 'Yes' at question '28 [TBI4]' (In your lifetime, have you ever injured your head or neck in a fight, from being hit by someone, or from being shaken violently? Have you ever been shot in the head?)

Please choose **only one** of the following:

- ○ No Loss of Conciousness (LOC)
- ○ Less than 30 minutes
- ○ 30 minutes to 24 hours
- ○ Greater than 24 hours

Were you dazed or did you have a gap in your memory from the injury? *

Only answer this question if the following conditions are met:
Answer was 'No Loss of Conciousness (LOC)' at question '30 [TBILOC3]' (How long were you knocked out or did you lose conciousness (LOC) for?)

Please choose **only one** of the following:

- ○ Yes
- ○ No

What age were you when this injury occured ?

Only answer this question if the following conditions are met:
Answer was 'Yes' at question '28 [TBI4]' (In your lifetime, have you ever injured your head or neck in a fight, from being hit by someone, or from being shaken violently? Have you ever been shot in the head?)

Only numbers may be entered in this field.

Please write your answer here:

In your lifetime, have you ever been nearby when an explosion or a blast occurred? If you served in the military, think about any combat- or training-related incidents. *

Please choose **only one** of the following:

- ○ Yes
- ○ No

What was the cause of the injury to your head or neck ?

Only answer this question if the following conditions are met:
Answer was 'Yes' at question '33 [TBI5]' (In your lifetime, have you ever been nearby when an explosion or a blast occurred? If you served in the military, think about any combat- or training-related incidents.)

Please write your answer here:

Please record only one response.

How long were you knocked out or did you lose conciousness (LOC) for? *

Only answer this question if the following conditions are met:
Answer was 'Yes' at question '33 [TBI5]' (In your lifetime, have you ever been nearby when an explosion or a blast occurred? If you served in the military, think about any combat- or training-related incidents.)

Please choose **only one** of the following:

- ○ No Loss of Conciousness (LOC)
- ○ Less than 30 minutes
- ○ 30 minutes to 24 hours
- ○ Greater than 24 hours

Were you dazed or did you have a gap in your memory from the injury? *

Only answer this question if the following conditions are met:
Answer was 'No Loss of Conciousness (LOC)' at question '35 [TBILOC4]' (How long were you knocked out or did you lose conciousness (LOC) for?)

Please choose **only one** of the following:

- ○ Yes
- ○ No

What age were you when this injury occured ?

Only answer this question if the following conditions are met:
Answer was 'Yes' at question '33 [TBI5]' (In your lifetime, have you ever been nearby when an explosion or a blast occurred? If you served in the military, think about any combat- or training-related incidents.)

Only numbers may be entered in this field.

Please write your answer here:

Have you ever had a period of time in which you experienced multiple, repeated impacts to your head (e.g. history of abuse, contact sports, military duty)? *

Please choose **only one** of the following:

- ○ Yes
- ○ No

What was the typical or usual effect from repeated head knocks? *

Only answer this question if the following conditions are met:
Answer was 'Yes' at question '38 [TBIrepeated]' (Have you ever had a period of time in which you experienced multiple, repeated impacts to your head (e.g. history of abuse, contact sports, military duty)?)

Please choose **only one** of the following:

- ○ Dazed or memory gaps
- ○ Loss of conciousness

What was the cause of the repeated injury to your head or neck ?

Only answer this question if the following conditions are met:
Answer was 'Yes' at question '38 [TBIrepeated]' (Have you ever had a period of time in which you experienced multiple, repeated impacts to your head (e.g. history of abuse, contact sports, military duty)?)

Please write your answer here:

Please record only one response.

What was the most severe effect from one of the times you had an impact to the head? *

Only answer this question if the following conditions are met:
Answer was 'Yes' at question '38 [TBIrepeated]' (Have you ever had a period of time in which you experienced multiple, repeated impacts to your head (e.g. history of abuse, contact sports, military duty)?)

Please choose **only one** of the following:

- ○ Dazed or Memory gap but No Loss of Conciousness (LOC)
- ○ Loss of conciousness for less than 30 minutes
- ○ Loss of conciousness for 30 minutes to 24 hours
- ○ Loss of conciousness for greater than 24 hours

Were you dazed or did you have a gap in your memory from the injury? *

Only answer this question if the following conditions are met:
Answer was 'Dazed or memory gaps' at question '39 [TBIRepeatedeffect]' (What was the typical or usual effect from repeated head knocks?)

Please choose **only one** of the following:

- ○ Yes
- ○ No

How old were you when these repeated injuries began and ended? *

Only answer this question if the following conditions are met:
Answer was 'Yes' at question '38 [TBIrepeated]' (Have you ever had a period of time in which you experienced multiple, repeated impacts to your head (e.g. history of abuse, contact sports, military duty)?)

	Head injuries began	Head injuries ended
Age		

Have you ever lost conciousness from a drug overdose or being choked? *

Please choose **only one** of the following:

- ○ Yes
- ○ No

Please indicate how many times you lost conciousness from a drug overdose or being choked:

Only answer this question if the following conditions are met:

Answer was 'Yes' at question '44 [TBI7]' (Have you ever lost conciousness from a drug overdose or being choked?)

	Overdose	Choked
Losses of consciousness	☐	☐

Lifestyle Behaviours

In the past year, have you used any of the following tobacco products? *

Please choose **all** that apply:

- ☐ No
- ☐ Cigarettes
- ☐ Cigars
- ☐ Pipes
- ☐ Smokeless tobacco (e.g. chewing, snuff, dip)

In the past 3 months, how often have you used the substances ? *

Please choose the appropriate response for each item:

	Never	Once or twice	Monthly	Weekly	Daily or almost daily
Tobacco products (cigarettes, chewing tobacco, cigars, etc.)	○	○	○	○	○
Alcoholic beverages (beer, wine, spirits, etc.)	○	○	○	○	○
Cannabis (marijuana, pot, grass, hash, etc.)	○	○	○	○	○
Cocaine (coke, crack, etc.)	○	○	○	○	○
Amphetamine type stimulants (speed, diet pills, ecstasy, etc.)	○	○	○	○	○
Inhalants (nitrous, glue, petrol, paint thinner, etc.)	○	○	○	○	○
Sedatives or Sleeping Pulls (Valium, Serepax, Rohypnol, etc.)	○	○	○	○	○
Halluginogens (LSD, acid, mushrooms, PCP, Special K, etc.)	○	○	○	○	○
Opiods (heroin, morphine, methadone, codeine, etc.)	○	○	○	○	○

Please specify any other substances not input above and the frequency of use:

Please write your answer here:

How often do you have a drink containing alcohol ? *

Please choose **only one** of the following:

- ○ Never
- ○ Monthly or Less
- ○ 2 to 4 times a month
- ○ 2 to 3 times a week
- ○ 4 or more times a week

In answering the following questions, please remember that a standard drink contains 10g of pure alcohol.

Only answer this question if the following conditions are met:
Answer was NOT 'Never' at question '49 [AUDIT_1]' (How often do you have a drink containing alcohol ?)

How many 'standard' drinks (see above) containing alcohol do you have on a typical day when you are drinking ? *

Only answer this question if the following conditions are met:
Answer was NOT 'Never' at question '49 [AUDIT_1]' (How often do you have a drink containing alcohol ?)

Please choose **only one** of the following:

- ○ 1 or 2
- ○ 3 or 4
- ○ 5 or 6
- ○ 7 to 9
- ○ 10 or more

Please select the response that best fits your drinking: *

Only answer this question if the following conditions are met:
Answer was NOT 'Never' at question '49 [AUDIT_1]' (How often do you have a drink containing alcohol ?)

Please choose the appropriate response for each item:

	Never	Less than monthly	Monthly	Weekly	Daily or almost daily
How often do you have six or more drinks on one occasion?	○	○	○	○	○

How often during the last 12 months have you found that you were not able to stop drinking once you had started?	○	○	○	○	○
How often during the last 12 months have you failed to do what was normally expected from you because of drinking?	○	○	○	○	○
How often during the last 12 months have you needed a drink in the morning to get yourself going after a heavy drinking session?	○	○	○	○	○
How often during the last 12 months have you had a feeling of guilt or remorse after drinking?	○	○	○	○	○
How often during the last 12 months have you been unable to remember what happened the night before because you had been drinking?	○	○	○	○	○

Have you or someone else been injured as a result of your drinking? *

Please choose **only one** of the following:

- ○ No
- ○ Yes, but not in the last 12 months
- ○ Yes, during the last 12 months

Has a relative, a friend, a doctor or other health professional been concerned about your drinking or suggested you cut down? *

Please choose **only one** of the following:

- ○ No
- ○ Yes, but not in the last 12 months
- ○ Yes, during the last 12 months

Do you presently have a problem with drinking? *

Please choose **only one** of the following:

- ○ No
- ○ Probably Not
- ○ Unsure
- ○ Possibly
- ○ Definitely

In the next 3 months, how difficult would you find it to cut down or stop drinking ? *

Please choose **only one** of the following:

- ○ Very Easy
- ○ Fairly Easy
- ○ Neither difficult nor easy
- ○ Fairly difficult
- ○ Very difficult
- ○ Not Applicable

{sum(AUDIT_1.value, AUDIT_2.value, sum(that.6.value),AUDIT_9.value, AUDIT_10.value)}

{sum(AUDIT_1.value, AUDIT_2.value, sum(that.6.sq_A.NAOK))}

{sum(that.6.sq_B.NAOK,that.6.sq_C.NAOK,that.6.sq_D.NAOK)}

On an average day, how many 250 - 375ml beverages containing caffeine do you drink (such as caffeine containing energy drinks, coffee, tea, coca-cola)? *

Please choose **only one** of the following:

- ○ None
- ○ 1-2 per day
- ○ 3-5 per day
- ○ 6-10 per day
- ○ 11 or more per day

How often do you currently take any of the following supplements? *

Please choose the appropriate response for each item:

	Never	Less than once a month	Monthly	Weekly	Daily or almost daily
Body building supplements (such as amino acids, weight gain products, creatine, etc.)	○	○	○	○	○
Energy supplements (such as energy drinks, pills, or energy enhancing herbs)	○	○	○	○	○
Weight loss supplements	○	○	○	○	○

Recent Health Symptoms - 1

Over the last 2 weeks, how often have you been bothered by any of the following problems? *

Please choose the appropriate response for each item:

	Not at all	Several days	More than half the days	Nearly every day
Little interest or pleasure in doing things	○	○	○	○
Feeling down, depressed, or hopeless	○	○	○	○
Trouble falling or staying asleep, or sleeping too much	○	○	○	○
Feeling tired or having little energy	○	○	○	○
Poor appetite or overeating	○	○	○	○
Feeling bad about yourself — or that you are a failure or have let yourself or your family down	○	○	○	○
Trouble concentrating on things, such as reading the newspaper or watching television	○	○	○	○
Moving or speaking so slowly that other people could have noticed? Or the opposite — being so fidgety or restless that you have been moving around a lot more than usual	○	○	○	○
Thoughts that you would be better off dead or of hurting yourself in some way	○	○	○	○

{sum(that.PHQ9.value)}

Recent Health Symptoms - 2

During the past 4 weeks, how much have you been bothered by any of the following problems ? *

Please choose the appropriate response for each item:

	Not Bothered at all	Bothered a little	Bothered a lot
Stomach pain	○	○	○
Back Pain	○	○	○
Pain in your arms, legs, or joints (knees, hips, etc.)	○	○	○
Menstrual cramps or other problems with your periods (Women Only)	○	○	○
Headaches	○	○	○
Chest pain	○	○	○
Dizziness	○	○	○
Fainting spells	○	○	○
Feeling your heart pounding or race	○	○	○
Shortness of breath	○	○	○
Pain or problems during sexual intercourse	○	○	○
Constipation, loose bowels, or diarrhea	○	○	○
Nausea, gas, or indigestion	○	○	○
Feeling tired or having low energy	○	○	○
Trouble sleeping	○	○	○

{sum(that.PHQ15.value)}

Recent Health Symptoms - 3

Over the last 2 weeks, how often have you been bothered by the following problems? *

Please choose the appropriate response for each item:

	Not at all	Several days	More than half the days	Nearly every day
Feeling nervous, anxious or on edge	○	○	○	○
Not being able to stop or control worrying	○	○	○	○
Worrying too much about different things	○	○	○	○
Trouble relaxing	○	○	○	○
Being so restless that it is hard to sit still	○	○	○	○
Becoming easily annoyed or irritable	○	○	○	○
Feeling afraid as if something awful	○	○	○	○

{sum(that.GAD.value)}

Recent Health Symptoms (Pain)

We are interested in learning more about your pain intensity and disability. For the following questions with a scale of 0-10, please place shade ONE circle only. Please complete these questions regardless of whether you have pain.

How would you rate your pain on a 0-10 scale at the present time, that is right now, where 0 is 'no pain' and 10 is 'pain as bad as could be'? *

Please choose the appropriate response for each item:

	0	1	2	3	4	5	6	7	8	9	10	
No Pain	○	○	○	○	○	○	○	○	○	○	○	Pain as bad as could be

In the past 6 months, on the average, how intense was your pain rated on a 0-10 scale where 0 is 'no pain' and 10 is 'pain as bad as could be'? (That is, your usual pain at times you were experiencing pain) *

Please choose the appropriate response for each item:

	1	2	3	4	5	6	7	8	9	10	
No Pain	○	○	○	○	○	○	○	○	○	○	Pain as bad as could be

About how many days in the last 6 months have you been kept from your usual activities (work, school or housework) because of pain? *

Your answer must be between 0 and 183
Only an integer value may be entered in this field.

Please write your answer here:

[ ]

days

In the past 6 months, how much has pain interfered with you daily activities rated on a 0-10 scale where 0 is 'no interference' and 10 is 'unable to carry on any activities'? *

Please choose the appropriate response for each item:

	1	2	3	4	5	6	7	8	9	10	
No interference	○	○	○	○	○	○	○	○	○	○	Unable to carry on any activities

In the past 6 months, how much has pain changed your ability to take part in recreational, social and family activities where 0 is 'nochange' and 10 is 'extremechange' ? *

Please choose the appropriate response for each item:

	1	2	3	4	5	6	7	8	9	10	
No Change	○	○	○	○	○	○	○	○	○	○	Extreme Change

In the past 6 months, how much has pain changed your ability to work (including housework) where 0 is 'no change' and 10 is 'extreme change'? *

Please choose the appropriate response for each item:

	1	2	3	4	5	6	7	8	9	10	
No	○	○	○	○	○	○	○	○	○	○	Extreme

change change

Please indicate below if you have had pain or tenderness over the past 7 days in any of the areas listed below. Be sure to mark right and left sides separately. *

Please choose the appropriate response for each item:

	Left	Right
Shoulder	○	○
Upper Arm	○	○
Lower Arm	○	○
Hip	○	○
Upper Leg	○	○
Lower Leg	○	○
Jaw	○	○
Chest	○	○
Abdomen	○	○
Upper Back	○	○
Lower Back	○	○
Neck	○	○
No pain in these areas	○	○

Your Health Now

In the past four (4) weeks: *

Please choose the appropriate response for each item:

	None of the time	A little of the time	Some of the time	Most of the time	All of the time
About how often did you feel tired out for no good reason ?	○	○	○	○	○
About how often did you feel nervous ?	○	○	○	○	○
About how often did you feel so nervous that nothing could calm you down ?	○	○	○	○	○
About how often did you feel hopeless ?	○	○	○	○	○
About how often did you feel restless or fidgety ?	○	○	○	○	○
About how often did you feel so restless you could not sit still ?	○	○	○	○	○
About how often did you feel depressed ?	○	○	○	○	○
About how often did you feel that everything was an effort ?	○	○	○	○	○
About how often did you feel so sad that nothing could cheer you up ?	○	○	○	○	○
About how often did you feel worthless ?	○	○	○	○	○

{sum(that.K1.value)}

The next few questions are about how these feelings may have affected you in the past four (4) weeks. You need not answer these questions if you answered 'None of the time' to all of the previous ten questions about your feelings. *

	Days
In the past four (4) weeks, how many days were you TOTALLY UNABLE to work, study or manage your day to day activities because of these feelings?	☐
[Aside from those days], in the past four (4) weeks, HOW MANY DAYS were you able to work or study or manage your day to day activities, but had to CUT DOWN on what you did days because of these feelings?	☐

*

	Times
In the past four (4) weeks, how many times have you seen a doctor or any other health professional about these feelings?	☐

In the past four (4) weeks, how often have physical health problems been the main cause of these feelings? *

Please choose **only one** of the following:

- ○ None of the time
- ○ A little of the time
- ○ Some of the time
- ○ Most of the time
- ○ All of the time

Past Experiences

Below is a list of problems and complaints that people sometimes have in response to stressful life experiences. Please read each one carefully, then select the circle to indicate how much you have been bothered by that problem in the last month. *

Please choose the appropriate response for each item:

	Not at all	A little bit	Moderately	Quite a bit	Extremely
Repeated, disturbing memories, thoughts, or images of a stressful experience from the past?	○	○	○	○	○
Repeated, disturbing dreams of a stressful experience from the past?	○	○	○	○	○
Suddenly acting or feeling as if a stressful experience were happening again (as if you were reliving it)?	○	○	○	○	○
Feeling very upset when something reminded you of a stressful experience from the past?	○	○	○	○	○
Having physical reactions (e.g., heart pounding, trouble breathing, or sweating) when something reminded you of a stressful experience from the past?	○	○	○	○	○
Avoiding thinking about or talking about a stressful experience from the past or avoid having feelings related to it?	○	○	○	○	○
Avoiding activities or situations because they remind you of a stressful experience from the past?	○	○	○	○	○
Trouble remembering important parts of a stressful experience from the past?	○	○	○	○	○
Loss of interest in things that you used to enjoy?	○	○	○	○	○
Feeling distant or cut off from other people?	○	○	○	○	○
Feeling emotionally numb or being unable to have loving feelings for those close to you?	○	○	○	○	○
Feeling as if your future will somehow be cut short?	○	○	○	○	○
Trouble falling or staying asleep?	○	○	○	○	○
Feeling irritable or having angry outbursts?	○	○	○	○	○
Having difficulty concentrating?	○	○	○	○	○
Being "super alert" or watchful on guard?	○	○	○	○	○
Feeling jumpy or easily startled?	○	○	○	○	○

{sum(that.PCL_C.value)}

Thankyou for undertaking this questionnaire. If you have any questions about what you have been asked, please contact Dr Scott Quadrelli (scott.quadrelli@newcastle.edu.au).

Questionnaire: All Groups

Questionnaire Version: 4

Date: 27/03/2014

Submit your survey.
Thank you for completing this survey.

Author Contribution Declaration

Six Fucose-α(1−2) Sugars and α Fucose Assigned in the Human Brain using In Vivo Two Dimensional Magnetic Resonance Spectroscopy at 3T.

Carolyn Mountford, Scott Quadrelli, Alexander Lin, Saadallah Ramadan.

Published: NMR in Biomedicine

Statement IV: Author Contribution

Author	Contribution to manuscript	Signature
Carolyn Mountford	Wrote and designed the manuscript, undertook critical review and made changes in response to the reviewer's comments.	
Scott Quadrelli	Wrote this the manuscript, data analysis, development of 3D visualisation tool for data, undertook critical review and made changes in response to the reviewer's comments.	
Alexander Lin	Data collection undertook critical review.	
Saadallah Ramadan.	Wrote manuscript and undertook critical review.	

_____

Prof Derek Lavar (Acting Assistant Dean Research Training)

Hitchhiker's Guide to Voxel Segmentation for Partial Volume Correction of In Vivo Magnetic Resonance Spectroscopy.

Scott Quadrelli, Carolyn Mountford, Saadallah Ramadan.

Published: NMR Insights
Statement IV: Author Contribution

Author	Contribution to manuscript	Signature
Scott Quadrelli	Wrote and designed the manuscript, undertook critical review and made changes in response to the reviewer's comments.	
Carolyn Mountford	Critical review of the manuscript.	
Saadallah Ramadan.	Contributed to the manuscript and undertook critical reviews.	

_____
Prof Derek Lavar (Acting Assistant Dean Research Training)

Systematic review of *in-vivo* Magnetic Resonance Spectroscopy for the assessment of Post-Traumatic Stress Disorder

Scott Quadrelli, Carolyn Mountford, Saadallah Ramadan.

Published: Psychiatric Research - Neuroimage

Statement IV: Author Contribution

Author	Contribution to manuscript	Signature
Scott Quadrelli	Wrote and designed the manuscript, undertook critical review and made changes in response to the reviewer's comments.	
Carolyn Mountford	Critical review of the manuscript.	
Saadallah Ramadan.	Contributed to the manuscript and undertook critical reviews.	

_____

Prof Derek Lavar (Acting Assistant Dean Research Training)

Neurochemical Dysregulation in Posttraumatic Stress Disorder identified using 1D and 2D-LCOSY spectroscopy: Preliminary Findings

Scott Quadrelli, Nathan Tosh, Aaron Urquhart, Katie Trickey, Rosanna Tremewan, Graham Galloway, Lisa Rich, Rod Lea, Peter Malycha, and Carolyn Mountford.

Under Review: Translational Psychiatry

Statement IV: Author Contribution

Author	Contribution to manuscript	Signature
Scott Quadrelli	Project design, data collecting, recruitment, data analysis and interpretation, writing and critical review of the manuscript.	
Nathan Tosh	Recruitment data collection, Psychological evaluation and manuscript preparation and review of manuscript.	
Aaron Urquhart	Data analysis.	
Katie Trickey	Psychological evaluation and manuscript preparation	
Rosanna Tremewan	Psychological evaluation and manuscript preparation	
Graham Galloway	Technical aspects of data collection and manuscript preparation	
Lisa Rich	Participant recruitment and initial clinical evaluation.	
Rod Lea	Statistical analysis.	
Peter Malycha	Oversight of the clinical criteria, recruitment and	

	critical review of the manuscript.	
Carolyn Mountford	Initial concept, project design, evaluation of data and writing and critical review of the manuscript.	

_____

Prof Derek Lavar (Acting Assistant Dean Research Training)

2D *in-vivo* L-COSY spectroscopy identifies neurometabolite alterations in multiple sclerosis, not identified using conventional 1D MRS.

Scott Quadrelli , Karen Ribbons, Jameen Arm, Oun Al-iedani, Jeannette Lechner-Scott, Rodney Lea, Saadallah Ramadan.

In preparation for: Radiology

Statement IV: Author Contribution

Author	Contribution to manuscript	Signature
Scott Quadrelli	Contributions to the technical design of the MRS methods, data analysis, interpretation of data, writing and critical revisions of the manuscript.	
Karen Ribbons	Project design, data collection, interpretation of data, writing and critical revision of manuscript.	
Jameen Arm	Data collection and analysis.	
Oun Al-iedani	Data collection and analysis.	
Jeannette Lechner-Scott	Project design, interpretation of data, writing and critical revision of manuscript.	
Rodney Lea	Statistical analysis, writing and critical revision of manuscript.	
Saadallah Ramadan.	Project design, interpretation of data, writing and critical revision of manuscript.	

_____

Prof Derek Lavar (Acting Assistant Dean Research Training)