New Approaches to the Use of Magnetic Resonance Spectroscopy for Investigating the Pathophysiology of Auditory-Verbal Hallucinations

Gerard Eric Dwyer

Thesis for the degree of Philosophiae Doctor (PhD)
University of Bergen, Norway
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Scientific environment

As a PhD candidate, I was employed at the Department of Biological and Medical Psychology at the University of Bergen, as a student of the International Graduate School in Integrated Neuroscience (IGSIN) at the Faculty of Psychology at the University of Bergen and a member of both the Bergen fMRI group, University of Bergen and the NORMENT Centre of Excellence, University of Oslo.

My supervisor for this project was Associate Professor Renate Grüner, who is affiliated with the Department of Radiology at Haukeland University Hospital, the Department of Physics and Technology at the University of Bergen and is the centre leader of the Mohn Medical Imaging and Visualisation centre. My co-supervisors for this project were Professor Kenneth Hugdahl, also affiliated with the Department of Biological and Medical Psychology at the University of Bergen and the NORMENT Centre of Excellence, University of Oslo as well as the Division of Psychiatry, Department of Clinical Medicine at the University of Bergen and the Department of Radiology at Haukeland University Hospital, and Chief Engineer Lars Ersland, affiliated with the Department of Clinical Engineering, Haukeland University Hospital. The research for this project was partly funded by grants to Kenneth Hugdahl from the Research Council of Norway, the European Research Council (ERC) and the Health Authority of Western Norway (Helse-Vest) to the Bergen fMRI group.

Acknowledgements

Through the four years spent on this PhD project, I'm very grateful to have had so many wonderful people around me as supervisors, co-authors, co-workers, colleagues, friends and family.

Two people I can never thank enough are my supervisor Renate Grüner, and co-supervisor Kenneth Hugdahl. During my Master's thesis project, where she was also my main supervisor, I remember apologising to Renate for spending an inordinate amount of time trying to find an answer to a rather trivial question, to which she simply said "Don't apologise for wanting to find answers to your questions, this is what makes you a good scientist" and I have not forgotten this. My thanks and my gratitude to Renate for her continued assistance and encouragement.

Kenneth Hugdahl, in addition to making this PhD project possible, and sending us around the world to soak our sponge-like brains in knowledge, has also been a great inspiration. Clarke's first law states "When a distinguished but elderly scientist states that something is possible, he is almost certainly right. When he states that something is impossible, he is very probably wrong." Listening to Kenneth, one feels as if anything is possible, they need only figure out how it needs to be done.

Clarke's third law states that "Any sufficiently advanced technology is indistinguishable from magic," by extension, Alex Craven is nothing short of a magician. Alex is the "sine qua non" of this project, and his invaluable work is greatly appreciated.

Massive thanks and gratitude go to everyone that helped in the planning, organisation, data collection and analysis involved with the three articles presented here, namely Renate Grüner, Kenneth Hugdahl, Karsten Specht, Alex Craven, Marco Hirnstein, Kristiina Kompus, Jörg Assmus, Lars Ersland, Justyna Bereśniewicz, and Katarzyna Kazimierczak.

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For the "chemistry" side of this project, thanks go to Tom Christian Adamsen and Ole Heine Kvernenes for helping me keep my lab stocked, and to John Georg Seland and Willy Nerdal with the NMR Spectroscopy group for collaborating with us and for helping me to (finally) understand what J-coupling is.

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Special thanks also to Karlijn Kouwer, the apprentice who became a master. Although the article that we had planned to write did not make it into this thesis, her contributions to our work on MR-spectroscopy are greatly appreciated.

To all the wonderful friends I've made, thank you for good times and happy memories including, but not limited to: a discussion about whether zebras are white with black stripes or black with white stripes, getting lost in Germany and finding ourselves in Switzerland, canoeing in Lake Constance, putting some *brom* in our *fiets* during our time in the Netherlands, and learning the difference between "oida" in Austrian and "oi da" in Norwegian. Thank you all for being your fine selves!

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Finally, to my wife Tone, a woman of great courage and great kindness who has seen me at my best and worst over the past four years and loves me all the same, thank you for your love, your support and for taking care of our little family.

Abstract

Magnetic resonance imaging (MRI) may be considered one of the most useful technological developments in the field of cognitive neuroscience. Above the ability to provide detailed anatomical images, MRI has afforded researchers new insights into both the healthy brain and pathological states through the ability to map neural activity with blood-oxygen-level dependent functional MRI (BOLD-fMRI) and to perform quantitative analyses of biochemical compounds *in vivo* with magnetic resonance spectroscopy (MRS).

Despite the usefulness of these methods, one of the limitations to BOLD-fMRI is that it does not reveal how changes in neural activity relate to excitation and inhibition in a neural circuit, commonly characterised as the excitation/inhibition balance. Similarly, while MRS may allow quantitative measurement of the primary excitatory and inhibitory neurotransmitters in the brain, namely glutamate and γ-aminobutyric acid (GABA), it may only reveal average levels over the course of an acquisition, and MRS alone provides no measure of neural activity.

A number of studies have attempted to relate patterns of activity as measured with fMRI with metabolite levels acquired separately with MRS, and though this approach may be useful for investigating how metabolite levels may predict or influence patterns of activity, it does not allow detection and measurement of acute metabolic changes occurring over short time scales, or how changes correlate with neural activity.

The motivation for this project was to investigate new approaches to performing MRS that would allow measurement of dynamic changes in the balance of excitatory and inhibitory neurotransmitter levels with high temporal resolution (i.e. functional MRS), as well as methods for simultaneous, functional imaging of both neural and metabolic activity. It is believed that these techniques may advance ongoing investigations within the research group into understanding of the pathophysiology of auditory-verbal hallucinations, one of the characteristic symptoms of schizophrenia.

This project proceeded through three key stages.

The first stage was to create an overview of key metabolites implicated in the pathophysiology of schizophrenia that may be measured with MRS. The first article in this thesis reviews current practice and new developments in the use of MRS in measuring metabolites in four categories: *N*-acetyl aspartate (NAA), glutamate and glutamine (or the composite "Glx" signal), GABA and glutathione (GSH).

The second stage was to evaluate a method for analysing MR-spectra in a time-resolved manner at high temporal resolution. Using transcranial direct current stimulation (tDCS) to induce a change in local levels of Glx and GABA, MR-spectra were acquired continuously before, during, and after stimulation and analysed using a novel "time-windowing" approach presented in the second article in this thesis.

Finally, the BOLD effect that provides the contrast used in BOLD-fMRI has been demonstrated to affect the linewidth of MR-Spectra. By acquiring spectra with interleaved frames without water suppression, changes in the linewidth of the unsuppressed water signal may be used as a measure of the BOLD effect, effectively permitting simultaneous measurement of neural and metabolic activity. The third article in this thesis was a feasibility study, determining whether this approach may be implemented using a MEGA-PRESS sequence at a field strength of 3 T in order to investigate how changes in local GABA and Glx levels relate to changes in the BOLD signal in response to visual stimulation.

The methods presented in this thesis show great potential for measuring dynamic changes in GABA levels as well as simultaneous measurement of neural and metabolic activity at a field strength of 3 T. However, more work is required to evaluate their effectiveness conclusively. Further studies may benefit from judicious choice of stimulus and experimental design as well as the use of specialised sequences that do not rely on spectral editing.

List of publications

I. Dwyer, G.E., Hugdahl, K., Specht, K. and Grüner, R. (2018). Current Practice and New Developments in the Use of In Vivo Magnetic Resonance Spectroscopy for the Assessment of Key Metabolites Implicated in the Pathophysiology of Schizophrenia. *Current Topics in Medicinal Chemistry,18* (21), 1908-1924.

II. Dwyer, G.E., Craven, A.R., Hirnstein, M., Kompus, K., Assmus, J., Ersland, L., Hugdahl, K. and Grüner, R. (2019). No Effects of Anodal tDCS on Local GABA or Glx Levels in the Left Posterior Superior Temporal Gyrus. *Frontiers in Neurology, 9,* 1145.

III. Dwyer, G.E., Craven, A.R., Bereśniewicz, J., Kazimierczak, K., Ersland, L., Hugdahl, K. and Grüner, R. Simultaneous measurement of the BOLD-effect and neurochemical changes in response to visual stimulation at 3 T. Manuscript submitted.

Reprints of "Current Practice and New Developments in the Use of in vivo *Magnetic Resonance Spectroscopy for the Assessment of Key Metabolites Implicated in the Pathophysiology of Schizophrenia" and "No Effects of Anodal tDCS on Local GABA or Glx Levels in the Left Posterior Superior Temporal Gyrus" are presented with permissions granted through open access publishing.*

Contents

1. Introduction

In understanding mental phenomena, Hugdahl and Sommer (2017) outline six levels of explanation at which they may be investigated, being the cultural, clinical, cognitive, imaging, cellular and molecular levels. At the cellular level, explanation is primarily concerned with the activity of neurons and neurotransmitters, and how the output of a neural circuit relates to input from excitatory and inhibitory postsynaptic potentials, commonly characterised as the excitation-inhibition (E/I) balance.

Impairments in the E/I balance have been implicated in range of neurological and psychiatric conditions, perhaps most notably schizophrenia, where converging evidence across genetic, physiological, post-mortem studies and animal models consistently find decreased inhibition and increased excitation in relation to both schizophrenia and auditory-verbal hallucinations (AVH), a defining symptom of the disease (Jardri et al., 2016).

Magnetic resonance imaging (MRI) provides two particularly useful tools for investigating mental phenomena at both the imaging and cellular levels, namely blood-oxygen-level dependent functional MRI (BOLD-fMRI or fMRI), in which neural activity is measured through associated changes in blood flow, and magnetic resonance spectroscopy (MRS) which may be used to perform quantitative measurements of glutamate and γ-aminobutyric acid (GABA), the principal excitatory and inhibitory neurotransmitters in the human brain respectively.

One of the limitations to fMRI is that it gives no indication of how changes in excitation and inhibition relate to neural activity. Evidence suggests that the measured signal is related to processing within a cortical region and more closely reflects input to a neural circuit rather than its output (Havlicek, Ivanov, Roebroeck, & Uludağ, 2017; Logothetis, Pauls, Augath, Trinath, & Oeltermann, 2001). However, as Logothetis (2008) illustrates, signal change may be due to a shift in either excitation or inhibition, or both, but cannot be deduced from fMRI alone.

Similarly, MRS may be used to perform quantitative measurements of brain metabolites involved in the E/I balance, such as glutamate and GABA, but alone gives no measure of neural activity. Furthermore, as it is typically used, MRS provides only a quantitative measurement averaged over the course of an acquisition that normally lasts several minutes and may not reveal metabolic changes occurring over short time scales.

Further investigation into mental phenomena at the cellular level depends on both the ability to measure dynamic changes in both glutamate and GABA at a temporal resolution comparable to that of fMRI, and to measure neural activity and associated changes in the E/I balance simultaneously. To this end, a number of studies have sought to overcome the limitations of fMRI and MRS by improving temporal resolution of MRS to perform time-resolved or functional MRS (Cleve, Gussew, & Reichenbach, 2015; Gussew et al., 2010; Lally et al., 2014), combining fMRI and MRS to investigate correlations between patterns of activity with resting-state metabolite levels (Falkenberg, Westerhausen, Specht, & Hugdahl, 2012; Hugdahl, Craven, et al., 2015) and by developing methods for measuring neural and metabolic activity simultaneously (Apšvalka, Gadie, Clemence, & Mullins, 2015; Ip et al., 2017).

The purpose of this project was to evaluate new approaches to MRS that may allow both improved temporal resolution of MRS and simultaneous measurement of neural and metabolic activity, with a specific focus on how they may be used in improving understanding of the pathophysiology of auditory-verbal hallucinations.

1.1 Functional Magnetic Resonance Imaging (fMRI)

1.1.1 Principles of Nuclear Magnetic Resonance

Nuclear Magnetic Resonance (NMR) refers to the physical phenomenon by which atomic nuclei absorb and re-emit electromagnetic radiation of a characteristic frequency. This phenomenon forms the basis for both magnetic resonance imaging and spectroscopy.

For an atomic nucleus to undergo NMR, there are certain criteria that must be met. In the context of NMR, atomic nuclei may be described by three key properties: the atomic number which gives the total number of protons, the mass number which gives the total number of protons and neutrons, and the spin quantum number (denoted "I"). The spin quantum number refers to the total angular momentum of a nucleus and may be either zero, where the number of protons and neutrons are both even, an integer where the number of protons and neutrons are both odd, or a half-integer where either the number of protons or neutrons is odd (Levitt, 2013).

Atomic nuclei possessing a non-zero spin acquire a magnetic moment, by which they produce a magnetic field. A nucleus with spin angular momentum (I) has an orientation associated with it (m_I), quantised such that for the value of I there are 2I+1 values of m_I that may take on a range of values between -I and +I in integer steps. For example, a nucleus with a spin angular momentum value of I = ½ has (2(½) + 1 = 2) two values of m_I associated with it, denoted +½ and -½, the difference between these values being an integer step of 1. In the presence of a strong, external magnetic field ($\mathbf{B}_0$), interactions between the magnetic moments of the nuclei and the external field create an energy difference between the two states described by Equation 1.1.

$$\Delta E = \gamma \hbar \boldsymbol{B_0} \tag{1.1}$$

Where γ is the gyromagnetic ratio, a constant of proportionality unique to each atomic nucleus that relates its angular momentum due to spin to the strength of its magnetic moment expressed in units of radians per second per Tesla (rad s^{-1} T^{-1}) and ħ is the reduced Planck constant (1.054571800(13) x 10^{-34} J·s rad^{-1}).

Under the influence of spin, the nuclear magnetic moments precess around the direction of the external field at an angular frequency corresponding to the difference in energy between the different values of m_I. This frequency of precession ($\boldsymbol{\omega}_0$) is proportional to the strength of the external magnetic field through the gyromagnetic ratio as expressed in the *Larmor Equation* (1.2).

$$\boldsymbol{\omega}_0 = -\gamma \boldsymbol{B}_0 \tag{1.2}$$

If electromagnetic radiation is applied at a frequency corresponding to the Larmor precession frequency, typically within the radiofrequency band (RF), this energy is absorbed and re-emitted by the sample. This process of absorption and emission at the Larmor frequency is termed *nuclear magnetic resonance.*

While all atomic nuclei with a non-zero spin may undergo resonance, further discussion will be confined to resonance of the ^{1}H nucleus which consists of a single proton and I = ½.

Being a spin-½ particle, the ^{1}H nucleus has two values of m_I (+½ and -½) which correspond to lower and higher energy states in the presence of an external magnetic field: Low Energy = +½ (parallel with $\mathbf{B}_0$), High Energy = -½ = (antiparallel with $\mathbf{B}_0$). The ratio of nuclei in each energy state can be described by a Boltzmann distribution (Equation 1.3).

$$\frac{High\ Energy}{Low\ Energy} = e^{\frac{-\Delta E}{kT}} \tag{1.3}$$

Where k is the Boltzmann constant (1.38064852(79) x 10^{-23} J K^{-1}) (Levitt, 2013), T is the absolute temperature in units Kelvin and $\Delta E = \gamma \hbar \mathbf{B}_0$ (Equation 1.1).

This distribution means that in a population of nuclei, there is a preference for the low energy state that is proportional to the strength of the external magnetic field. Although the magnetic moments of nuclei in the high and low energy states cancel one another out, this slight excess of nuclei in the low energy state creates a net nuclear magnetisation that forms a measurable signal from the sample. Thus, it is not

individual nuclei, but rather the coherence of an ensemble of nuclei in the low energy state that forms a measurable signal described as the net magnetisation vector (NMV).

At rest, the NMV is oriented parallel to the external field $\mathbf{B}_0$, typically taken as the longitudinal or z-direction, with individual nuclei precessing at the Larmor frequency. When exposed to a pulse of electromagnetic radiation equal to the Larmor frequency, referred to as an excitation pulse or RF pulse (denoted $\mathbf{B}_1$), the NMV moves into the transverse plane (x-y plane). The angle made between the longitudinal and transverse planes is referred to as the flip angle (α) and is dependent on the strength of the applied RF pulse and its duration (τ) (Equation 1.4).

$$\alpha = \gamma |\boldsymbol{B}_1| \tau \tag{1.4}$$

Where τ is the duration of the applied pulse. Given that the gyromagnetic ratio and $\mathbf{B}_1$ frequency are constants dependent on the nucleus in which resonance is to be induced, the flip angle is largely determined by the duration of the pulse (τ). MRI scanners are designed such that signals are detected in the transverse plane, when net magnetisation is orthogonal to $\mathbf{B}_0$, so the signals are strongest when the flip angle is 90°. When $\mathbf{B}_1$ is removed, magnetisation comes under the influence of the main field $\mathbf{B}_0$ and returns to its low energy state, parallel with the external field in the longitudinal direction, under two related but independent relaxation processes: T1 and T2 relaxation.

T1 relaxation, also known as spin-lattice relaxation, is the process by which nuclei release the energy they have absorbed into the surrounding environment or molecular lattice. The flip angle closes and the magnetisation vector returns from the transverse plane to the longitudinal. The rate of recovery is an exponential process where the T1 time represents how long it takes for 63% of the longitudinal magnetisation to recover.

T2 relaxation, also known as spin-spin relaxation is the process by which the magnetic fields of nearby nuclei interact, leading to dephasing, loss of coherence, and

loss of magnetisation in the transverse plane. This loss of coherence is also an exponential process where the T2 time represents how long it takes for 63% of the transverse magnetisation to be lost. Another related process is that of T2* relaxation, which refers to dephasing and loss of coherence due to inhomogeneities in the main magnetic field.

The Larmor equation (Equation 1.2) establishes that resonance frequency is proportional to the strength of the magnetic field nuclei experience, as a result, resonance frequency may be manipulated by introducing gradients in the external magnetic field to localise resonance to spatial locations commonly referred to as *voxels* (from volumetric pixel, i.e. volumetric picture element). Resonance frequency within a voxel may be used to encode its spatial position in imaging, or be used to identify compounds of interest in spectroscopy.

The manipulation of the magnetic moments of MR-active nuclei between the longitudinal and transverse planes is accomplished through the coordination of RF pulses in combination with magnetic field gradients, the magnitude and timing of which are determined by *pulse sequences*. The three key elements of pulse sequences are the flip angle (Equation 1.4), the echo time (TE), which is the time between the application of an initial excitatory RF pulse and the peak of signal readout, and the repetition time (TR), which is the time between successive excitation pulses in an acquisition.

Manipulation and measurement of nuclear magnetic resonance through different pulse sequences effectively forms the basis of both magnetic resonance imaging and spectroscopy.

1.1.2 Blood-Oxygen-Level Dependent (BOLD) Contrast

Functional magnetic resonance imaging (fMRI) refers to methods allowing visualisation of changes in activity or metabolism over time as opposed to static images. Though not the only form of functional imaging, the term fMRI typically refers to blood-oxygen-level dependent (BOLD) fMRI, which uses the magnetic

properties of haemoglobin, the protein that binds oxygen in red blood cells, to relate changes in neural activity in areas of the brain to associated changes in blood flow to that area.

Haemoglobin is the protein responsible for transporting oxygen in red blood cells. The protein is comprised of four subunits called heme groups, each of which contains a single iron atom, to which oxygen atoms bind. The binding of oxygen to iron changes its magnetic properties such that oxygenated haemoglobin (oxyhaemoglobin) is diamagnetic and will repel magnetic fields, whereas deoxygenated haemoglobin (deoxyhaemoglobin) is paramagnetic and will attract magnetic fields.

Neural activity is marked by increases in energy-demanding processes and consequently, increases in oxygen and glucose consumption. The demand for glucose and oxygen and supply through local cerebral blood flow (CBF) are very tightly connected, such that as neural activity increases, CBF increases proportionally. This change in blood flow in response to neural activity is referred to as the haemodynamic response (HDR), and the relationship between neural activity and changes in CBF referred to as neurovascular coupling. Despite the proportional connection between neural activity and CBF, the rate of metabolic glucose consumption is greater than that of oxygen consumption, leading to an increase in the local fraction of oxyhaemoglobin relative to deoxyhaemoglobin in the local cerebral blood volume (CBV) (Fox & Raichle, 1986). Over the course of three landmark papers published in 1990 (Ogawa & Lee, 1990; Ogawa, Lee, Kay, & Tank, 1990; Ogawa, Lee, Nayak, & Glynn, 1990), Seiji Ogawa and associates demonstrated that these changes could be measured and imaged with MRI. Increases in the fraction of oxy- to deoxyhaemoglobin create less inhomogeneity in the local magnetic field, reducing dephasing due to T2* effects and increasing local MR-signal intensity. Thus, increases in neural activity may be imaged through increases in MR-signal due to increases in the ratio of oxy- to deoxyhaemoglobin as a result of increased CBF.

As the BOLD signal reflects changes in CBF and not neural activity directly, there is a delay between the onset of neural activity and the HDR of around 1-2 seconds, with

the HDR peaking around 4-6 seconds after activation and falling slightly under baseline before returning. A model of the haemodynamic response function (HRF) is depicted in Figure 1.

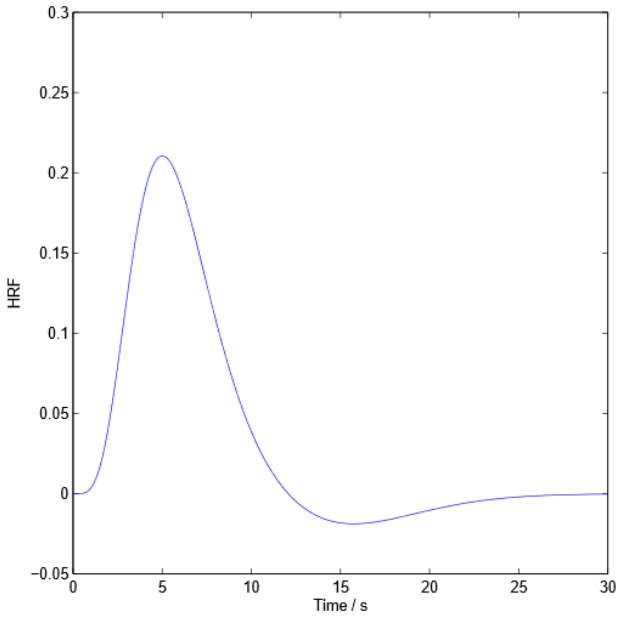

Figure 1 - Haemodynamic Response Function (HRF) in response to neural activation at time = 0 s

BOLD-fMRI experiments typically involve conditions where participants are exposed to a stimulus or perform a sensory, motoric or cognitive task alternating with periods of either no task or a different task or stimulus. The volumetric images acquired in fMRI may be compared between stimulus/task and rest/control conditions, or between different conditions, and testing for statistically significant changes in voxel intensity to create a map of areas where changes in neural activity where measured (Figure 2).

Condition presentation typically falls into one of three categories: Block designs, where epochs of one condition are presented for a certain duration alternating with either a control or other experimental condition; event-related designs, where a

condition is presented and the individual haemodynamic response to that task or stimulus measured; or mixed designs, in which an event-related design is presented in blocks (Amaro & Barker, 2006).

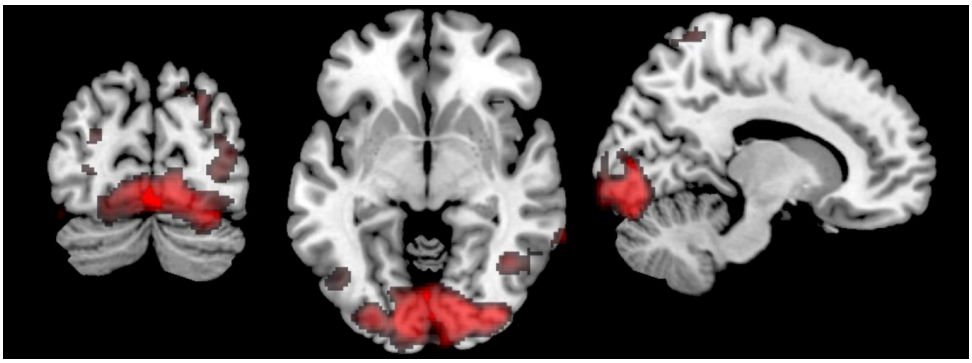

Figure 2 - Coronal (left), axial (middle) and sagittal (right) fMRI images showing activation in the visual cortex (red). Activation map shows contrast between blocks of visual stimulation with a red-black radial checkerboard alternating at 8 Hz and control blocks without stimulation (taken from article III).

1.1.3 Neural Activity and fMRI

The fundamental function of an individual neuron is the generation of an action potential, which may be considered the "output" of the neuron. The initiation of an action potential is determined by "inputs" received from other neurons to which it forms synaptic connections. Inputs may be excitatory, increasing neuronal potential towards the threshold for an action potential, or inhibitory, decreasing the potential from the threshold. Although well over 100 compounds have been identified and classified as neurotransmitters (Kalat, 2019) the primary excitatory and inhibitory neurotransmitters in the brain are glutamate and GABA respectively.

As many as 85% of the synapses in the brain use glutamate for transmission (Agarwal & Renshaw, 2012). In addition to its role as a neurotransmitter, glutamate is also involved in energy metabolism, protein synthesis and as a precursor to other important biomolecules (e.g. GABA and glutathione).

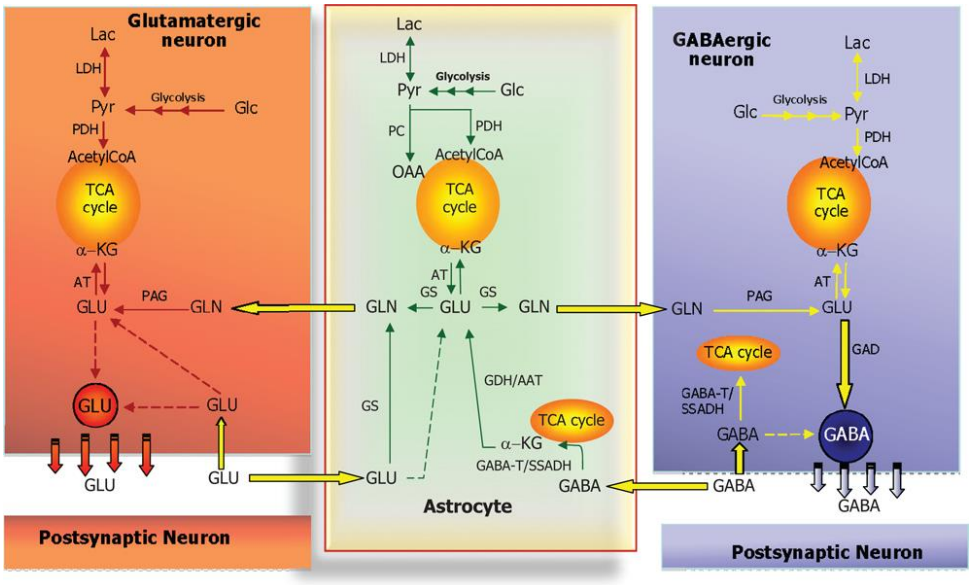

Figure 3 - Metabolism of glutamate and GABA. AAT = aspartate aminotransferase, AcetylCoA = Acetyl Coeznyme A, AT = aminotransferase, GABA = γ-aminobutyric acid, GABA-T = GABA transaminase, GDH = glutamate dehydrogenase, Glc = glucose, Gln = glutamine, Glu = glutamate, GS = glutamine synthase, Lac = lactate, LDH = lactate dehydrogenase, OAA = oxaloacetate, PAG = phosphate-activated glutaminase, PDH = pyruvate dehydrogenase, Pyr = pyruvate, SSADH = succinic semialdehyde dehydrogenase, TCA = tricarboxylic acid, α-KG = α-ketoglutarate. (Schousboe, Bak, & Waagepetersenm 2013, reprinted with permission).

As the blood-brain barrier prevents glutamate from entering neurons directly, it is produced locally by two separate pathways: *de novo* synthesis in the presynaptic neuron and recycling through astrocytes (Figure 3). *De novo* synthesis begins with glucose, which enters cells and is passed through the glycolytic pathway to produce pyruvate, which is in turn converted to acetyl-coenzyme A (acetyl-coA) and passed into the tricarboxylic acid cycle where it leaves as α-ketoglutarate which is converted by amino acid aminotransferases to glutamate. Following binding to postsynaptic receptors, glutamate is actively transported to astrocytes where it is converted to glutamine in a reaction catalysed by glutamine synthase, and may be recycled back to the presynaptic neuron to be converted to glutamate in a reaction catalysed by

phosphate-activated glutaminase. The uptake of glutamate by astrocytes also stimulates glycolysis and increases lactate production within the astrocytes (Pellerin & Magistretti, 1994). Glutamatergic transmission, including synthesis, release and recycling, is a highly energetically demanding process, and accounts for 70-80% of glucose consumption in glutamatergic neurons (Agarwal & Renshaw, 2012; Schousboe et al., 2013).

The synthesis of GABA in GABAergic neurons begins with glutamate, which may either be synthesised *de novo* following the same reaction pathway as in glutamatergic cells, or may be derived from glutamine supplied by astrocytes which is converted to glutamate by phosphate-activated glutaminase. With a glutamate substrate available, GABA is synthesised in a single step by the enzyme glutamic acid decarboxylase, only found in GABAergic cells, which removes a carboxyl group from glutamate to produce GABA (Agarwal & Renshaw, 2012). Following binding to postsynaptic receptors, may undergo reuptake into the presynaptic neuron and reused, or may be transported into astrocytes, converted to glutamine and returned to the presynaptic terminal in the same fashion as glutamate (Brady, Siegel, Albers, & Price, 2012).

Together, glutamatergic and GABAergic neurons in the cerebral cortex comprise neuronal microcircuits, which are defined by Silberberg, Grillner, LeBeau, Maex, and Markram (2005) as the "minimal number of interacting neurons that can collectively produce a functional output" (Figure 4). Both glutamatergic and GABAergic cells in cortical microcircuits receive input via long-range axons originating from excitatory neurons in subcortical regions (e.g. the thalamus) as well as from different cortical regions and layers. Interactions between excitatory glutamatergic cells and inhibitory GABAergic cells within a microcircuit are reciprocal, glutamatergic cells both excite and are inhibited by GABAergic cells, and form feedback and feedforward inhibitory loops (Isaacson & Scanziani, 2011). The output of a neural microcircuit is determined by the amount of excitatory and inhibitory activity within the circuit, commonly characterised as the excitation/inhibition (E/I) balance.

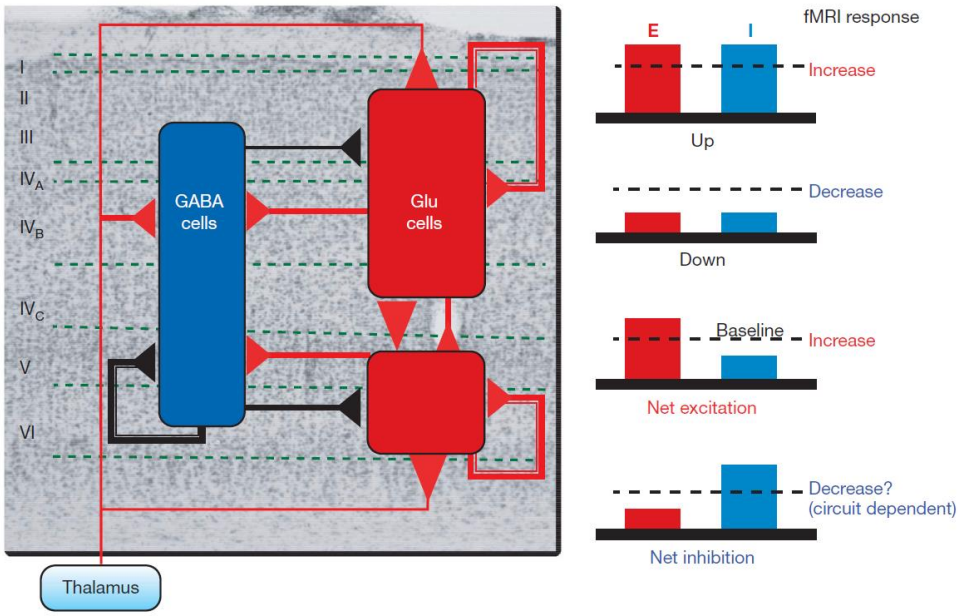

Figure 4 - Left: Model of a canonical cortical microcircuit showing excitatory (red) and inhibitory (black) synapses. Right: fMRI response to changes in excitation (red) and inhibition (blue) (Logothetis, 2008, reprinted with permission).

Given that the BOLD signal reflects changes in CBF due to neurovascular coupling, and that the haemodynamic response is related to increased demand for glucose and oxygen as a result of increased energy expenditure, it is important to distinguish what this energy demand indicates at a neurophysiological level. Converging evidence, mainly from electrophysiological studies in animal subjects (Logothetis et al., 2001; Mathiesen, Caesar, Akgören, & Lauritzen, 1998; Rauch, Rainer, & Logothetis, 2008; Viswanathan & Freeman, 2007), suggests the increased energy expenditure the BOLD signal represents is more closely related to the energy-demanding processes related to synaptic activity, integrative processing and the synthesis, release and recycling of neurotransmitters (i.e. input) rather than the generation of action potentials (i.e. output) of a neuronal microcircuit. As Viswanathan and Freeman (2007) demonstrate, stimuli able to elicit synaptic activity without initiating an action

potential in the primary visual cortex of cats still resulted in changes in tissue oxygen concentration.

In an article entitled “What we can do and what we cannot do with fMRI”, (Logothetis, 2008) demonstrates that changes in the fMRI signal may be due to shifts in excitation or inhibition, or both, but cannot be deduced from fMRI alone (Figure 4). To this end, magnetic resonance spectroscopy may offer a potential solution to the limitations of fMRI, allowing quantitative measurements of glutamate and GABA *in vivo* and bridging the gap between imaging neural activity and underlying changes in the E/I balance.

1.2 Magnetic Resonance Spectroscopy (MRS)

Magnetic Resonance Spectroscopy is an MR-imaging technique that allows non-invasive quantitative analyses of biochemical compounds to be performed *in vivo.* The principles of nuclear magnetic resonance that form the basis of imaging also form the basis of spectroscopy, with the exception that in imaging, resonance frequency is used to encode a position in space, whereas in spectroscopy it is used as a marker for identifying compounds based on their molecular environment.

A Fourier transform of the measured resonance signals produces an MR-spectrum which is a plot of resonance frequency, typically along the x-axis, against intensity (Figure 5a) where “peaks” in the spectrum correspond to nuclei resonating at that frequency, and the integral area of each peak is proportional to their abundance.

1.2.1 Chemical Shift and J-Coupling

Electrons surrounding atomic nuclei produce magnetic fields of their own, small in comparison to the external field, but strong enough to augment the effective magnetic field nuclei experience and consequently, their resonance frequency. Thus, the actual magnetic field nuclei experience is affected by the density of electrons surrounding them. The electrons are said to “shield” the nucleus, and the nuclear resonance frequency may be shifted depending on how much shielding electrons provide against

the external field. The presence of other atoms (e.g. fluorine, chlorine) or functional groups (e.g. amine groups, carboxyl groups) in a molecule will withdraw electrons away from hydrogen atoms, proportionally decreasing their shielding and increasing their resonance frequency. This shift in resonance is known as chemical shift (δ) and maybe be used to identify compounds based on characteristic resonance frequencies of nuclei indicative of their local molecular environment. Chemical shift is typically reported as the difference between the resonance of the signal (ν_{sample}) and a reference signal ($\nu_{reference}$) divided by the resonance frequency of the reference signal at a given field strength (Equation 1.5).

$$\delta = (\nu_{sample} - \nu_{reference}) / \nu_{reference} \quad (1.5)$$

As the difference between two signals is given in the range of Hz, divided by the resonance frequency of the reference signal in MHz, chemical shift is typically expressed in units of parts-per-million (ppm), i.e. Hz per MHz. The advantage to this system of measurement is that the frequency of $\nu_{reference}$ and the difference between ν_{sample} and $\nu_{reference}$ vary proportionally with field strength, giving chemical shift the same value in units of ppm regardless of the field strength of the scanner used.

In addition to chemical shift, MR-active nuclei may also experience the effects of J-coupling, also known as scalar or spin-spin coupling, wherein nuclei influence the resonance frequencies of one another through hyperfine interactions between the nucleus and surrounding electrons. Where the spin state of one nucleus affects the resonance frequency of another nucleus in the same molecule, the two nuclei are said to be "coupled" to one another. Coupling between nuclei in a molecule will cause minor shifts in resonance frequency. Averaged out over the whole sample, the resonance peak will appear to be "split" into (n+1) sub-peaks, where n is the number of other nuclei a nucleus is coupled to. Unlike chemical shift, J-coupling is not field strength dependent, and J-coupling constants are typically reported in Hz. Figure 5c illustrates how while chemical shift remains constant, the distance between sub-peaks due to splitting is reduced at higher field strengths.

Figure 5b depicts a simulated MR-spectrum of GABA at 3 T demonstrating the effects of chemical shift and splitting due to J-coupling. The three peaks correspond to hydrogen atoms on the three methylene groups (CH_2) in GABA, coloured red, blue and green. The amine group (NH_4^+) exerts a stronger electron withdrawing influence over the molecule than the carboxyl group (COO^-), causing a larger chemical shift in the "red" methylene group (3.0 ppm) than the "blue" (2.3 ppm) or "green" (1.9 ppm) groups. The two red hydrogen nuclei are J-coupled to the two blue, causing the red peak to be split into a triplet of three sub-peaks. Similarly, the two green hydrogens are split by the blue into a triplet. The blue hydrogens are coupled to both the red and green and are split into a quintet. Despite splitting, as each peak corresponds to two hydrogen nuclei, the integral area of each resonance multiplet is the same (De Graaf, 2007; Puts & Edden, 2012).

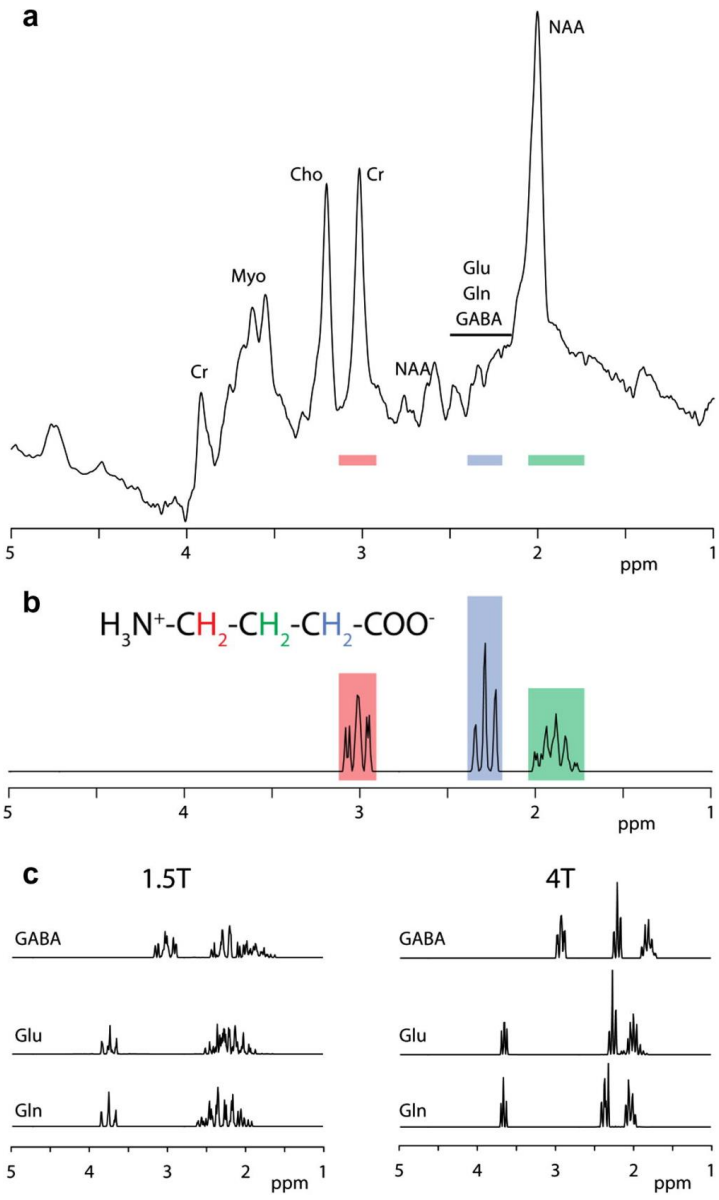

Figure 5 – a) MR-spectrum of the human brain acquired at 3T, showing peaks corresponding to N-acetyl aspartate (NAA), creatine (Cr), choline (Cho), myo-inositol (Myo), glutamate (Glu), glutamine (Gln) and GABA. Coloured bars correspond to the peak locations in (b). b) MR-spectrum of GABA at 3T, showing resonance from the three methylene groups in the GABA molecule. c) Simulated MR spectra of GABA, Glu and Gln at 1.5 and 4 T (Puts & Edden, 2012, reprinted with permission).

1.2.2 Metabolite Quantification

The integrated area of a spectral peak is proportional to the number of nuclei resonating at that frequency and may be used to derive quantitative estimates of the abundance of particular metabolites. For quantitative analyses, simple approaches of numerical integration and calculating the area under peaks cannot distinguish contributions from overlapping or poorly resolved peaks (Stagg & Rothman, 2014). Figure 5a and 5c illustrate how overlap in the major resonance signals from glutamate, glutamine and GABA contribute to the same peak in an MR-spectrum, making it difficult to determine what fraction of each metabolite contributes to the measured peak. One of the most commonly used methods for spectral quantification is basis spectrum fitting, in which a spectrum is analysed as a linear combination of model spectra from individual metabolite sources.

Basis spectrum fitting requires a set of model metabolite spectra that may either be simulated or acquired *in vitro* from single metabolite spectroscopy phantoms to produce a "basis set" of spectra acquired or simulated with the same acquisition parameters as the spectrum to be analysed. From this, a model of the spectrum under analysis may be reconstructed as a linear combination of the basis spectra of metabolites present in the sample to fit the measured spectrum, accounting for residual noise and variation in the spectral baseline (Figure 6).

Using complete model spectra as opposed to individual resonance peaks allows the maximum amount of prior information to be incorporated into the analysis and the complexity of individual spectra may be used to resolve overlapping peaks at one chemical shift if their peaks are separated elsewhere in the spectrum.

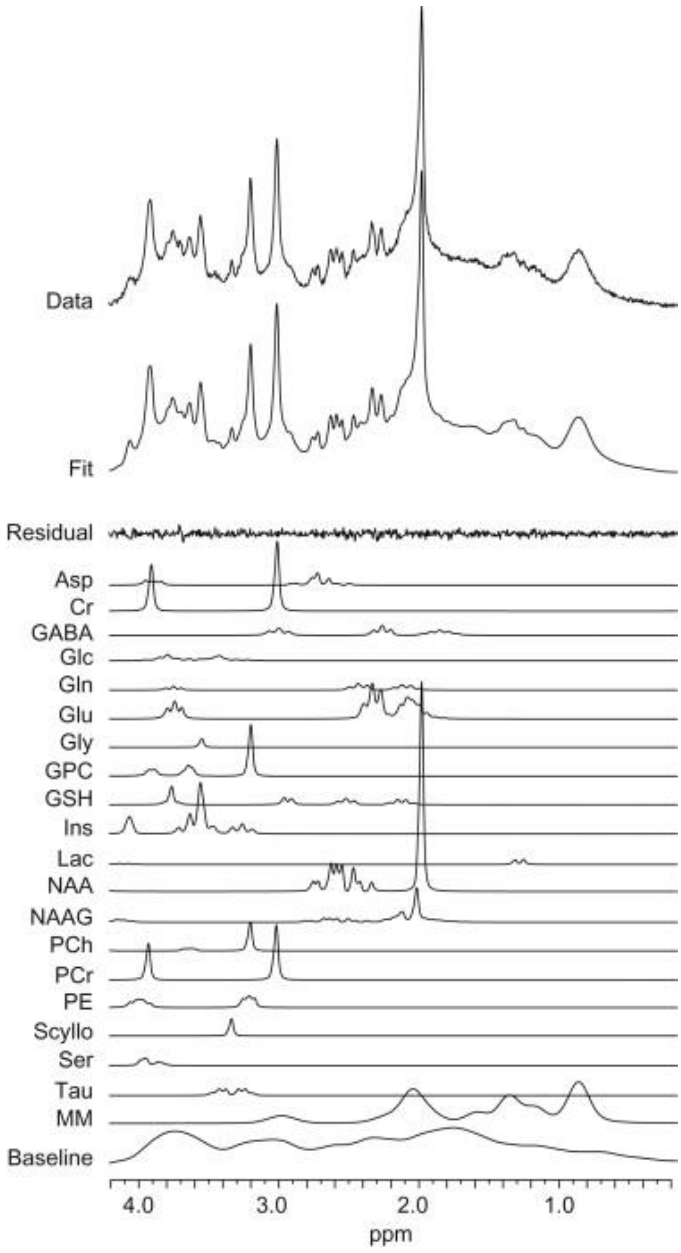

Figure 6 - Basis spectrum fitting. The measured spectrum (Data) is modelled as a linear combination of the basis spectra (Fit). The difference between the fit and data forms the residual which may be used indication of how well the model matches the data. (Stagg & Rothman, 2014, reprinted with permission).

Quantitative metabolite estimates from MR-spectra are determined relative to a reference signal. Metabolites may be reported as a ratio relative to another metabolite, most commonly creatine, which has been demonstrated to be relatively stable under normal physiological conditions (Stagg & Rothman, 2014) or water, which is typically measured from spectral frames acquired without water suppression during the spectral acquisition. Given sufficient information regarding the water composition of the spectroscopy voxel and relaxation times of metabolites of interest, an estimation of absolute molar (moles per unit volume, e.g. mol L^{-1}) or molal (moles per unit mass, e.g. mol kg^{-1}) concentration may be derived, but this estimate is still based on the ratio of metabolite peak areas relative to that of tissue water in the spectroscopy voxel (Danielsen & Henriksen, 1994; Kreis, Ernst, & Ross, 1993).

1.2.3 Pulse Sequences and Spectral Editing

As with imaging, the coordination of excitation pulses and magnetic gradients are used to induce resonance within a volume of interest (voxel), typically between 1 and 27 mL in volume, with the size of the voxel being proportional to spectral SNR. Two of the most widely used pulse sequences for MRS are the PRESS (Point REsolved Spectroscopy Sequence) and STEAM (STimulated Echo Acquisition Mode) sequences. The key difference between these two sequences is with the PRESS sequence, two 180° pulses are used to restore full coherence in the transverse plane, giving a greater SNR than with a STEAM sequence. Alternatively, the advantage with a STEAM sequence is that coherence is restored with 90° pulses, permitting shorter echo times than those that 180° pulses necessitate, allowing signals to be recovered from compounds of interest with short T2 times (Figure 7).

In a standard *in vivo* MRS acquisition, the strongest measurable signals come from the two hydrogen nuclei present in water, which resonate as a singlet at 4.63 ppm. With a concentration of around 43.4 moles L^{-1} (M) in grey matter and 36.0 M in white matter, water has a concentration between 10^3 and 10^4 times higher than most metabolites of interest (Stagg & Rothman, 2014). In order to improve detection of the weaker signals from less abundant metabolites, spectral acquisition typically

incorporates a water suppression step to reduce the water signal. The most common approach to water suppression involves a CHEmical Shift Selective 90° excitation pulse (CHESS pulse) designed to selectively excite water signals at around 4.63 ppm. With the water proton signals already in the transverse plane, the first excitation pulse of the spectroscopy sequence pushes these signals a further 90° into the longitudinal plane in the opposite direction, minimising their detection in the transverse plane.

Though a wide range of metabolites may be detected with PRESS and STEAM sequences, many compounds of interest may be difficult to detect due to factors such as low biological concentrations and overlap in spectral profiles with other, stronger signals. As figure 5c illustrates, separation between glutamate, glutamine and GABA may be difficult, particularly at lower field strengths (i.e. ≤ 3 T), due to significant spectral overlap between 2.0 and 2.5 ppm. Due to poor resolution between resonance signals in this region, quantitative estimates are often reported as "Glx", a composite signal of glutamate and glutamine with small contributions from GABA and glutathione (GSH) (De Graaf, 2007).

Accurate detection and quantification of certain metabolites of interest may be possible through the use of specialised sequences, such as the MEGA (MEscher-GArwood) spectral editing method (Mescher, Merkle, Kirsch, Garwood, & Gruetter, 1998; Mescher, Tannus, Johnson, & Garwood, 1996). MEGA editing exploits the unique modulation of MR-signals due to J-coupling, and how increasing echo time may change the spectral profile of coupled nuclei. One of the most widely used applications of MEGA editing is for the detection of GABA at field strengths of 3 T and lower, typically implemented in a PRESS sequence as MEGA-PRESS. At an echo time of 68 ms, the outer peaks of the triplet that GABA normally produces at 3.0 ppm are inverted (Figure 8b). By applying a selective refocusing pulse at 1.9 ppm between the second and third RF pulses (Figure 7), the outer triplet peaks may be reverted. By interleaving spectral frames both with ("ON") and without ("OFF") the refocusing pulse, the two sets of ON and OFF spectra may be paired up and subtracted from one another to produce a difference spectrum in which GABA may be visualised at 3.0 ppm (Figure 8).

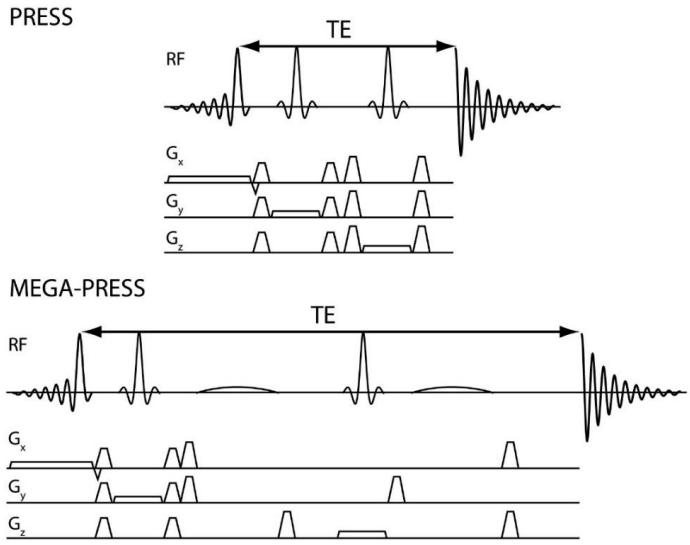

Figure 7 - Pulse sequence diagrams for the PRESS (top) and MEGA-PRESS (bottom) sequences. RF = Radiofrequency pulse, G_x, G_y and G_z indicate applied gradients in the x, y and z directions respectively. TE = Echo Time (Mullins et al., 2014, reprinted with permission).

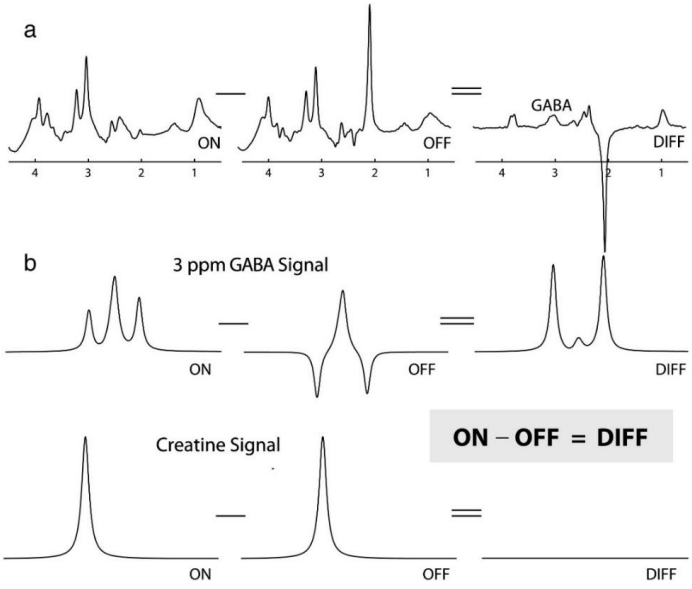

Figure 8 - Schematic for MEGA-PRESS. a) Subtracting the OFF spectra from ON produces the difference spectrum. b) Subtracting "negative" peaks in the OFF spectra creates "positive" peaks in the difference spectrum. Uncoupled signals, and those not affected by editing pulses (e.g. creatine) are removed in the subtraction (Mullins et al., 2014, reprinted with permission).

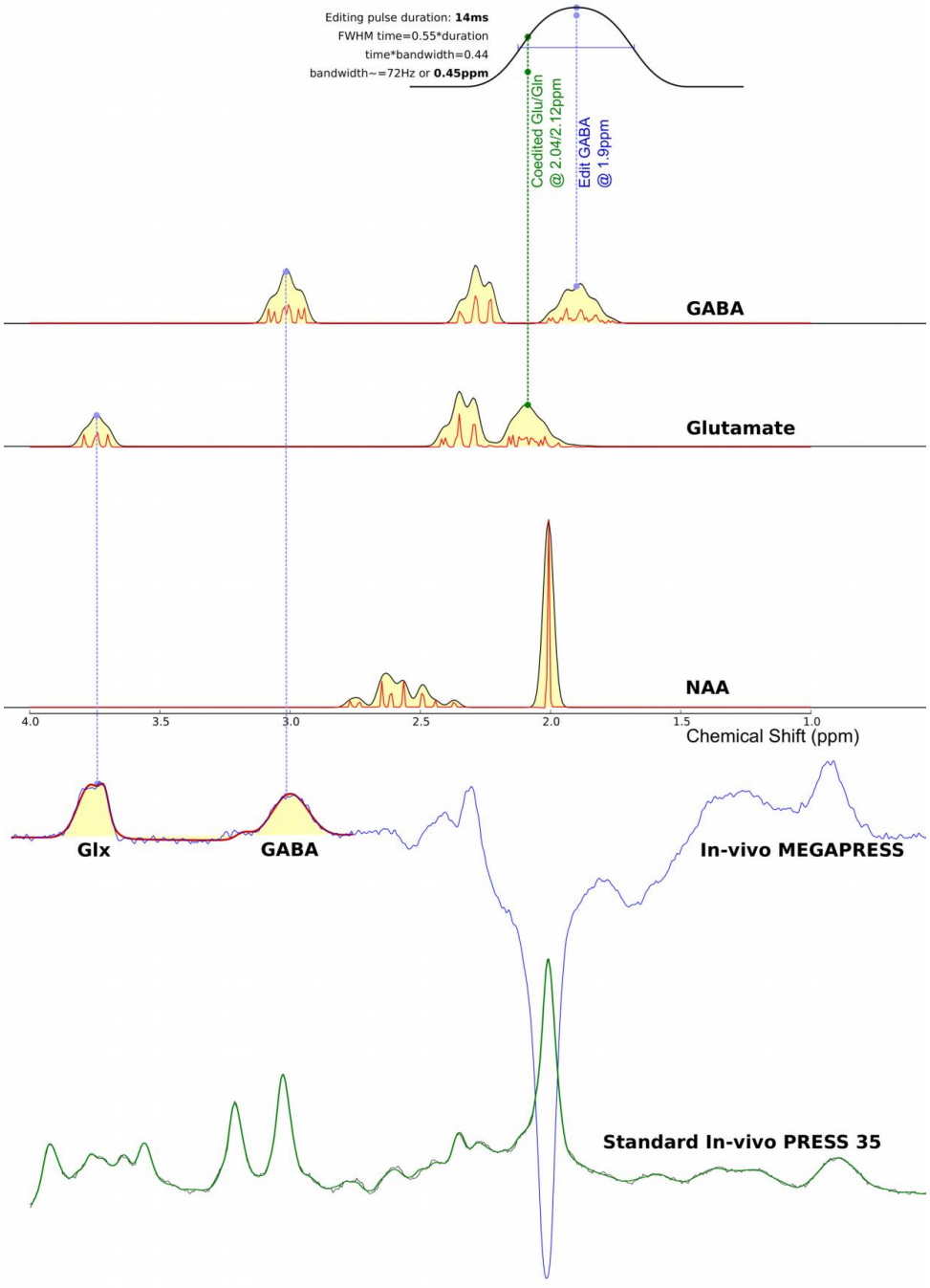

Figure 9 - How editing pulses affect coupled signals (Craven, 2018, reprinted with permission).

The editing pulse used to "correct" the inverted peaks on the GABA triplet at 1.9 ppm also affects the large NAA resonance signal at 2.0 ppm, leading to a large negative peak in this area in the difference spectrum. This pulse also affects resonances attributed to the Glx peak at 2.04 and 2.12 ppm, coupled to resonances that also appear as a peak in the difference spectrum at 3.7 ppm (Figure 9). Thus, the difference spectrum contains resonances from Glx, GABA and NAA from which quantitative estimates may be derived, comparable in terms of accuracy and reliability to standard short echo time PRESS sequences (Henry, Lauriat, Shanahan, Renshaw, & Jensen, 2011).

One issue affecting MEGA-edited GABA spectroscopy is the co-editing of macromolecule (MM) resonances at 1.7 ppm contaminating the GABA signal in the difference spectrum. Thus, GABA as measured with MEGA-PRESS typically refers to both GABA and the co-edited macromolecule, denoted GABA+MM or simply GABA+, unless macromolecular suppression has been explicitly implemented (Edden, Puts, & Barker, 2012).

The SNR of a single spectral acquisition is often too low for meaningful data to be extracted from it. Typical practice involves acquiring a number of spectral frames that are averaged together to produce a "time-averaged" spectrum, wherein SNR increases proportionally with square root of the number of averages incorporated into the spectrum (Barker, Bizzi, De Stefano, Gullapalli, & Lin, 2009). Total scan times typically range from at least three to five minutes, depending on repetition time and the nature of the experiment, and it follows that total acquisition time must be longer when performing spectral editing to obtain a comparable SNR. Thus, quantitative analysis of a time-averaged spectrum provides the concentration of measured metabolites over the course of the entire acquisition.

1.2.4 Functional MRS

Performing MRS in a time-resolved or functional manner, i.e. functional MRS (fMRS), involves the acquisition and analysis of spectra in such a way that dynamic changes in metabolite levels may be measured in relation to a stimulus, task or

change in state. In what is widely considered the first fMRS experiment, Prichard et al. (1991) investigated a mismatch between increased blood flow and glucose uptake with oxygen consumption in the visual cortex in response to visual stimulation (Fox, Raichle, Mintun, & Dence, 1988). Spectra were acquired over an 80 minute period, during which 48 minutes of photic stimulation was delivered through a set of goggles containing a 5 x 6 grid of red light-emitting diodes flashing at 16 Hz. Spectra were combined to produce time-averaged spectra incorporating 128 averages each, representing blocks of 6-8 minutes of the total acquisition, such that an updated measure of lactate levels could be made at 6-8 minute intervals. This approach revealed that the mismatch between blood flow and glucose uptake with oxygen consumption could be attributed to a transient excess of glycolysis over aerobic respiration as indicated by an increase by 0.3-0.9 mM in lactate levels in the visual cortex during the first few minutes of stimulation which declined as stimulation continued.

Subsequent fMRS studies have been used to measure increases in glutamate and lactate in the occipital cortex (OCC) in response to visual stimulation (Bednařík et al., 2015; Lin, Stephenson, Xin, Napolitano, & Morris, 2012; Mangia et al., 2007; Mekle et al., 2017; Schaller, Mekle, Xin, Kunz, & Gruetter, 2013), changes in glutamate in the anterior cingulate cortex (ACC) and insula in response to pain (Cleve, Gussew, Wagner, Bär, & Reichenbach, 2017; Gussew et al., 2010; Gutzeit et al., 2011; Mullins, Rowland, Jung, & Sibbitt, 2005) as well as dynamic changes in GABA in the sensorimotor cortex in response to learning (Floyer-Lea, Wylezinska, Kincses, & Matthews, 2006) and in the dorsolateral prefrontal cortex (DLPFC) under a working memory task (Michels et al., 2012).

The common approach to functional MRS has been to acquire spectra in serial blocks incorporating a small number of averages (Floyer-Lea et al., 2006; Ip et al., 2017; Kupers, Danielsen, Kehlet, Christensen, & Thomsen, 2009; Michels et al., 2012; Mullins et al., 2005; Rowland et al., 2005) effectively trading off a decrease in signal-to-noise ratio (SNR) for an increase in temporal resolution. However, this approach is best suited for spectroscopy at higher field strengths, i.e. > 3 T, where the SNR is

inherently higher and blocks may be made shorter. Furthermore, temporal resolution with this approach is still in the order of minutes, significantly longer than what can be achieved with BOLD-fMRI, and averaging over long stimulation blocks may not reveal metabolic events occurring on a shorter time scale.

More recent attempts to perform functional spectroscopy on a time scale comparable to that of BOLD-fMRI have used an event-related style of experimental design, in which a spectrum is recorded for each repetition of an experimental condition and taken together to produce an averaged spectrum of the metabolic response (Apšvalka et al., 2015; Cleve et al., 2015; Gussew et al., 2010; Lally et al., 2014). Using this approach, it may also be possible to alter the time of stimulus onset with the spectral acquisition in such a way that a metabolic response curve may be produced on a very short time scale, a process known as "jittering" used extensively in BOLD-fMRI (Mullins, 2018).

1.2.5 Combining fMRI and MRS

Despite the strengths of functional MRS, one of its limitations is that it may not measure neural activity directly. While it is possible to perform an experimental paradigm using fMRI and fMRS separately (Cleve et al., 2017), attempts have been made to measure both activity and metabolism simultaneously using a combination of fMRI and fMRS.

Ip et al. (2017) used a combined fMRI/MRS paradigm to investigate dynamic relationships between the BOLD signal and local glutamate levels. Within a single TR of 4000 ms, both a full brain 3D BOLD echo-planar imaging (EPI) volume and an MR-spectra from a 20 x 20 x 20 mm^3 voxel in the OCC were acquired. Using this approach, an increase in BOLD-signal (1.43 ± 0.17%) and glutamate levels (0.15 ± 0.05 I.U. ~2%) was able to be measured in the OCC in response to 64 seconds of visual stimulation in the form of a radial checkerboard alternating at 8 Hz.

Another approach to combining BOLD-fMRI and MRS takes advantage of the BOLD-effect on the lineshape of MR-Spectra. Shortly after the development of

BOLD-fMRI, Hennig, Emst, Speck, Deuschl, and Feifel (1994) investigated the nature of the BOLD-effect using a PRESS spectroscopy sequence without water suppression to examine BOLD related changes to the lineshape of the water resonance signal during neural activation. Given the high SNR of the unsuppressed water signal, and the high temporal resolution permissible where no time-averaging is required, this study proposed fMRS without water suppression as a possible alternative to BOLD-fMRI on systems where appropriate sequences could not be implemented (Hennig, 2012). Using this method, Frahm, Krüger, Merboldt, and Kleinschmidt (1996) found that in response to visual stimulation, measurements of the BOLD effect in the OCC produced almost identical time courses whether measured with fMRI or fMRS of the unsuppressed water resonance. Zhu and Chen (2001) found that in response to 12.8 minutes of visual stimulation the local BOLD-effect had affected both increased the height (H) and decreased the linewidth, ($\upsilon_{1/2}$), of the spectral peaks of NAA (H: 2.5%, $\Delta\upsilon_{1/2}$: −1.7%), creatine (H: 3.1%, $\Delta\upsilon_{1/2}$: −1.8%) and water (H: 3.1%; $\Delta\upsilon_{1/2}$: −2.3%) at 4 T.

Based on this principle, Apšvalka et al. (2015) investigated the possibility of measuring neural activity and metabolic changes simultaneously using only fMRS by interleaving spectral frames with and without water-suppression, providing an updated water signal from which BOLD-related changes could be derived. In this experiment investigating the relationship between dynamic changes in glutamate levels and BOLD effects in a repetition suppression paradigm, stimuli were presented in a mixed block/event-related design, with each 9.6 minute acquisition comprising 8 blocks of 12 trials lasting 3 seconds each. Stimulus presentation was coupled to spectral acquisition such that a single frame was acquired with each trial. Collecting spectra in this manner allowed the data to be analysed either as a block design, grouping and averaging spectra according to the block type, or as an event-related design, grouping and averaging frames by presentation type. On average, glutamate levels increased after novel visual stimuli presentations by 12% and decreased by 11–13% on repeated compared to novel presentations. The BOLD signal showed significant difference between task and rest trials, and a significant difference

between the novel and repeated trials, but appeared not to be connected to glutamate levels.

The results of Apšvalka et al. (2015) showed that simultaneous functional imaging of neural activity and biochemical changes could be performed with only simple modification to a standard spectroscopy sequence.

1.3 Schizophrenia and Auditory Verbal Hallucinations

Schizophrenia comprises a spectrum of disorders defined by the diagnostic and statistical manual of mental disorders 5th edition (DSM-V) as schizophrenia, schizotypal (personality) disorder and other psychotic disorders, all of which are defined by abnormalities in one or more of the following domains: delusions, hallucinations, disorganised thinking, grossly disorganised or abnormal motor behaviour (including catatonia) and negative symptoms. The diagnostic criteria for schizophrenia include abnormalities in at least two of these domains, with at least one of them being delusions, hallucinations or disorganised speech, each present for a significant portion of time during a 1-month period (American Psychiatric Association, 2013).

1.3.1 Biological Bases of Schizophrenia

Although the neurobiological mechanisms underlying schizophrenia are not fully understood, Lisman (2012) suggests that dysfunction in glutamatergic, GABAergic and dopaminergic neurotransmitter systems may be implicated in its pathophysiology.

The implication of glutamatergic systems follows from two key observations: Firstly, that glutamate receptor inhibitors, specifically N-methyl-D-aspartate receptor (NMDA-R) antagonists such as PCP, ketamine and related drugs, can elicit schizophrenia-like psychotic symptoms in healthy individuals (Javitt, Zukin, Heresco-Levy, & Umbricht, 2012). Secondly, administration of ketamine under

laboratory conditions was shown to exacerbate symptoms in schizophrenia patients (Lahti, Koffel, LaPorte, & Tamminga, 1995).

The implication of GABA stems from early post-mortem studies that found significantly lower concentrations of GABA in the thalamus and nucleus accumbens of schizophrenia patients compared to controls (Perry, Buchanan, Kish, & Hansen, 1979). Furthermore, Guidotti et al. (2000) found a 30% to 50% decrease in expression of genes coding glutamic acid decarboxylase, the enzyme catalysing GABA synthesis from glutamate, in the prefrontal cortex and cerebellum of patients with schizophrenia or bipolar disorder with psychosis, but not in those with unipolar depression without psychosis, compared to control subjects.

Dysfunction along dopaminergic pathways is one of the core neurobiological elements of schizophrenia and was initially informed by two key observations: Firstly, that the use of dopamine releasing stimulants such as amphetamines and cocaine could elicit some of the positive symptoms of schizophrenia in otherwise healthy individuals and exacerbate symptoms in schizophrenia patients. Furthermore, long term use of such substances may produce a drug-induced psychosis symptomatically similar to the positive symptoms of schizophrenia (Curran, Byrappa, & Mcbride, 2004). Secondly, first-generation antipsychotics such as chlorpromazine that were effective in alleviating positive symptoms of schizophrenia were shown to have inhibitory or antagonistic effects on dopamine receptors, more specifically on the D2 subtype (Chandler, 2015).

Due to the heterogeneity of the diagnostic criteria for schizophrenia and the diversity in symptoms the disease presents with, investigations into the biological bases of the disease are complicated, and many research groups have instead chosen to focus on a particular dimension or single symptom of the disease (Hugdahl, 2015). To this end, many symptom-focused studies have investigated auditory-verbal hallucinations, and in particular, functional imaging of auditory-verbal hallucinations.

1.3.2 Investigating Auditory-Verbal Hallucinations with MRI

An auditory-verbal hallucination (AVH) is the conviction that one is perceiving a "voice" in the absence of a corresponding auditory source (Hugdahl, 2015; McCarthy-Jones et al., 2014) commonly referred to as the experience of "hearing voices". Hugdahl (2017) outlines three core dimensions that define the experience of AVHs in schizophrenia patients: Firstly, there is a perceptual dimension, the voice "speaks" to the patient. Secondly, there is an attentional dimension, the patient cannot "control" the voice, and there is an associated impairment in perception of and attention to external sounds when the patient attends the voice. Finally, there is an emotional dimension: The voices are often perceived as having a negative emotional valence which, combined with diminished sense of executive cognitive control in the patient, may lead to the patient developing a sense of the voice having a "controlling" effect.

fMRI studies into AVHs suggest that they are an anomaly of speech perception rather than speech production, although speech production areas are also involved to some extent (Sommer et al., 2008). Converging evidence from separate meta-analyses showed spontaneous activity in speech perception areas in the left superior temporal gyrus (STG) and superior temporal sulcus (STS) and that these patterns of activity closely resemble patterns of activity measured in healthy individuals when listening to speech from an external source (Hugdahl, 2017; Jardri, Pouchet, Pins, & Thomas, 2011; Kompus, Westerhausen, & Hugdahl, 2011; Kühn & Gallinat, 2012; van den Noort, Specht, Rimol, Ersland, & Hugdahl, 2008). However, during AVHs, spontaneous activity has also been observed in the right prefrontal cortex, believed to be related to the semantic simplicity and negative emotional valence typically associated with AVHs in schizophrenia patients (Hugdahl, 2017; Jardri et al., 2011; Sommer et al., 2008).

One of the particularly interesting aspects of AVHs that fMRI has illuminated is the "paradoxical" finding that schizophrenia patients show increased activation both in the left primary auditory cortex and right rostral prefrontal cortex in the absence of an

external auditory stimulus and *decreased* activation in the presence of an external auditory stimulus (Kompus et al., 2011). Ćurčić-Blake et al. (2017) suggests that this apparent paradox may be explained by the so-called "saturation" theory, and that hallucinations create competition for neurophysiological resources common to the experience of hallucinations and the perception of external speech.

In a study specifically investigating the neurochemistry of AVHs in schizophrenia patients, Hugdahl, Craven, et al. (2015) acquired spectra from four locations: the left and right upper posterior temporal lobes, covering Heschl's gyri and the primary auditory cortices, and the left and right inferior frontal gyri in the frontal lobe, of healthy controls and schizophrenia patients. The patient group, however, was subdivided into frequent and non-frequent hallucinating subjects based on their hallucinatory behaviour (P3) score on the Positive and Negative Syndrome Scale (PANSS) (Kay, Fiszbein, & Opler, 1987). While schizophrenia patients as a group exhibited lower Glx levels than healthy controls, increased levels were found in patients with severe and frequent hallucinations compared to non-frequent, a finding replicated, in essence, by Ćurčić-Blake et al. (2017) suggesting that while glutamatergic transmission may be compromised in schizophrenia patients, glutamate levels may be a mediating factor in the experience of auditory verbal hallucinations.

Hugdahl (2017) raises a simple, yet often overlooked question regarding auditory-verbal hallucinations and their spontaneity, that is, why do patients not constantly hear voices? Why do they come and go? Considering this, the question becomes not only one of what causes AVHs, but what causes them to stop? Glutamatergic, dopaminergic and GABAergic pathways have been implicated in the pathogenesis of AVHs, but could the experience of hallucinations be caused by excess excitation or insufficient inhibition? Furthermore, does the cessation of hallucinations represent the re-establishing of the excitation/inhibition balance? Or possibly the exhaustion of resources? The ability to perform simultaneous measurement of neural activity and glutamate and GABA levels, at a temporal scale comparable to the spontaneity of AVHs, may potentially provide answers to these questions.

2. Specific Background and Aims of the Study

The purpose of this project was to evaluate methods allowing improved temporal resolution of MRS and methods for measuring neural and metabolic activity simultaneously, with a focus on application in the investigation of auditory-verbal hallucinations (AVHs). Understanding the neurochemical bases of AVHs is the focus of a number of ongoing research projects at the Bergen fMRI group, and the motivation for this project. It is believed that these methods may assist in understanding events occurring at the "imaging" and "cellular" levels of explanation.

Article I

The first article in this thesis is a review of current practice and new developments in the use of MRS for quantitative measurement of metabolites implicated in the pathogenesis of schizophrenia. This review focused on metabolites in four categories: *N*-acetyl aspartate (NAA), glutamate and glutamine (or the composite signal denoted Glx), GABA, and glutathione (GSH), covering evidence for their roles in the pathogenesis of the disease and issues related to their detection and measurement with MR-spectroscopy.

Article II

The primary aim of this study was to investigate the biological effects of transcranial direct current stimulation (tDCS) in the posterior superior temporal gyrus (pSTG), a method that has been investigated as a potential treatment for AVHs. The secondary aim was to test a novel approach to analysing MR-spectra in a time-resolved manner. tDCS was used to induce a change in local GABA and Glx levels in the pSTG. Spectra were acquired continuously before, during and after ten minutes of stimulation and analysed using a "time-windowing" method, allowing dynamic imaging of biochemical changes with very high temporal resolution.

Article III

The purpose of this study was to determine feasibility for simultaneous imaging of neural activity and dynamic changes in GABA at 3 T. The BOLD effect that provides the contrast in BOLD-fMRI has also been demonstrated to affect the linewidth in MR-spectra, and may be used as an indirect measure of neural activity. To determine whether it may be possible to measure neural and metabolic activity simultaneously, spectroscopy was performed with interleaved frames with and without water-suppression, providing an updated water reference from which a measurement of the BOLD effect could be derived from the linewidth of the water peak in the unsuppressed spectrum. Spectra were acquired from the occipital cortex of healthy participants undergoing visual stimulation in the form of an alternating, radial, red-black checkerboard.

3. Methods

3.1 Literature Review

Between the 13th and 30th of May 2018, a series of searches were performed in Pubmed to collect material for the review using the search term "MRS Spectroscopy" in conjunction with "Schizo*" (116 results), and "Psychosis" (54 results). Given its place as one of the defining symptoms of schizophrenia, and that significant work has focused on the biological basis of hallucinations with schizophrenia patients included amongst subjects, the term "Hallucinations" (6 results) was also included. As the review was to cover current practices, a restriction was placed confining results to those published in the last five years.

To be considered for inclusion in the review, articles were to use ^{1}H SVS methods, leading to the exclusion of studies using ^{13}C or ^{31}P spectroscopy as well as Chemical Shift Imaging (CSI) and MRS-Imaging (MRSI) methods. The studies had to focus on human subjects with schizophrenic or psychotic patients comprising at least one of the subject groups, to the exclusion of studies using animal models and animals as subjects as well as studies focusing on compounds of interest in healthy subjects only. Finally, articles had to be original research articles and not reviews or meta-analyses.

Following exclusion of articles not meeting the inclusion criteria and articles common to multiple searches, the review of current practice comprised 41 original research articles that were then separated into four groups according to major findings concerning the metabolites of interest: 1) NAA, 2) Glutamate and/or Glutamine, 3) GABA and 4) GSH. Studies reporting findings affecting more than one of these metabolites of interest were grouped according to the finding deemed most significant or relevant relative to the experimental hypothesis.

3.2 Participants

All studies involving human participants were performed in accordance with the recommendations and ethical approval of the regional committee for medical health and research ethics (REK-Vest) (Article II case number 2013/2342, Article III case number 2016/1629) with written informed consent from all participants.

The participant group in article II comprised 20 healthy participants (mean age: 25 years, range: 19 – 32; 10 male). All participants were required to complete a Norwegian language version of the Edinburgh handedness inventory (Oldfield, 1971) in order to determine right-handedness and to control for issues related to lateralisation of cortical areas, such that stimulation in the left hemisphere affects approximately the same functional area in each participant. The test assessed dominance of right and left hand in performing 10 everyday activities to produce a score ranging between -100 (exclusively left handed) and +100 (exclusively right handed), participants with a score greater than +40 were considered to be right handed and were permitted into the study (mean score: +80, SD: 24). Based on self-report, participants were free from psychiatric and neurologic conditions and had not used any psychoactive/psychotropic substances, including no smoking or other tobacco based or nicotine containing products, for six months prior to participating in the experiment. Participants were also instructed not to consume alcohol for at least 24 hours prior to participation. Data from one female participant was omitted from final analyses due to abnormally high measurements of Glx more than three standard deviations above the group mean (Glx levels almost five times higher than average values), suggesting an error in spectral acquisition.

The participant group in article III comprised 20 healthy participants (mean age: 29, range: 20-40; 11 male). Based on self-report, participants were free from psychiatric and neurological conditions, and not currently using any psychoactive/psychotropic substances.

3.3 Stimulation

In order to evaluate methods for measuring dynamic changes in neural and metabolic activity, it was necessary to induce such changes by means of stimulation. In article II, transcranial direct current stimulation (tDCS) was used in an attempt to increase local levels of glutamate and decrease local GABA levels in the posterior superior temporal gyrus (pSTG). In article III, visual stimulation in the form of a red and black radial checkerboard, alternating at 8 Hz, was used to increase neural activity in the occipital cortex (OCC), eliciting a BOLD response, increasing local levels of glutamate and possibly decreasing local GABA levels.

3.3.1 Transcranial Direct Current Stimulation

Transcranial direct current stimulation is a non-invasive neurostimulation technique used to modulate cortical excitability in a polarity dependent manner, with anodal stimulation typically increasing excitability and cathodal stimulation decreasing excitability (Zaghi, Acar, Hultgren, Boggio, & Fregni, 2010). Previous studies have shown that anodal tDCS may reduce local GABA levels in the motor cortex (Kim, Stephenson, Morris, & Jackson, 2014; Stagg et al., 2009) and increase local Glx and NAA levels in the intraparietal and prefrontal cortices (Clark, Coffman, Trumbo, & Gasparovic, 2011; Hone-Blanchet, Edden, & Fecteau, 2016).

Although the purpose of this experiment was to test a spectral analysis method, and any form of stimulation, task or activity that could increase activity in a region of interest in which an MRS voxel could be placed could have been used, tDCS in the pSTG was chosen for two reasons: Firstly, previous studies have shown tDCS to induce changes in local Glx and GABA levels and secondly, tDCS in the pSTG has been investigated as a potential treatment for AVHs in schizophrenia patients with studies finding both positive (Brunelin et al., 2012; Homan et al., 2011; Mondino et al., 2016) and negative results (Fitzgerald, McQueen, Daskalakis, & Hoy, 2014; Fröhlich et al., 2016). The stimulation paradigm and the stimulation parameters used were chosen in light of the potential for this study to advance not only neuroimaging,

but the understanding of tDCS as a potential treatment for AVHs (Brunelin et al., 2012; Fitzgerald et al., 2014; Fröhlich et al., 2016; Mondino et al., 2016).

tDCS was performed using an MR-compatible DC-Stimulator MR (neuroConn GmbH, Ilmenau, Germany) fitted with two 5 x 7 cm (35 cm^2) MR compatible rubber electrodes. The anodal electrode was placed with the centre of the pad on an area over the pSTG, such that the lower corners of the 7 cm edge of the electrode touched points T3 and T5 in the EEG 10-20 system. The cathodal electrode was placed over the contralateral orbitofrontal cortex, a site commonly used in tDCS montages for placement of the reference electrode (Nitsche et al., 2008; Nitsche & Paulus, 2000) such that the centre of the electrode covered point AF8 in the EEG 10-20 system. Each electrode was coated with a layer of Ten20 conductive paste (Weaver and Company, Aurora, United States of America) at the interface between electrode and skin to improve both adhesion and conductivity.

The study described in article II followed a stratified, randomised, double-blind design, with both participants and experimenters blind to the stimulation condition. Each subject participated in two MR-scanning sessions with tDCS, one with active and one with sham stimulation, separated by a wash-out period of one hour outside of the scanner, counterbalanced for order. Double-blinding was performed by having the stimulation condition determined by a code, independently predetermined by a researcher not present at the stimulation, such that each participant underwent both active and sham stimulation conditions and that an equal number of participants experienced active and sham stimulation as the first condition.

Once the electrodes were in place, participants were placed in the scanner with electrodes attached but not connected to the stimulation box. Electrodes were then connected prior to the spectroscopy acquisition. Once ten minutes of spectroscopy had elapsed, stimulation was initiated at the control box located outside the scanner at the control room using the appropriate code. Active stimulation was delivered for ten minutes with 24 seconds of ramping time both before and after the stimulation period at a constant intensity of 2.0 mA. For the sham stimulation condition, intensity was

ramped up to 2.0 mA over 24 seconds, then delivered for another 40 seconds before being ramped down to zero, giving participants a similar sensation to that they would experience during active stimulation.

3.3.2 Visual Stimulation

Early investigations into the relationship between neural activity and metabolism with positron emission tomography (PET) used visual stimulation to induce activity in the primary visual areas in the occipital cortex (OCC), a stimulus-response pair known to elicit consistent visual-evoked potentials (Fox & Raichle, 1984, 1985). Subsequent fMRI studies established that visual stimulation produced an easily detectable increase in the intensity of the MR-signal localised to the OCC (Kwong et al., 1992; Ogawa et al., 1992). As described in section 1.2.4 of this thesis, visual stimulation has also been the stimulus of choice for many investigations into metabolic changes associated with changes in neural activity. The ability for visual stimulation to produce a consistent and robust response in both neural activity and metabolism in a known region of interest made it an ideal choice for the assessment of the modified MRS sequence used in article III.

A radial red and black checkerboard pattern alternating at a frequency of 8 Hz was used as the visual stimulus, a stimulation paradigm previous studies demonstrated to induce both measurable neural activity and metabolic changes in the OCC (Ip et al., 2017; Mangia et al., 2006; Mangia et al., 2007).

The work of Fox & Raichle (1984, 1985) found that stimulus rate frequency had a greater effect on the magnitude of change in cerebral blood flow than stimulus luminance, and there was a linear relationship between stimulus frequency and change in cerebral blood flow peaking at around 7.8 Hz and declining thereafter, making 8 Hz the optimal frequency for an alternating stimulus to induce the largest BOLD response. A red and black pattern, such as that used by Mangia et al. (2006) was used rather than high contrast black and white as it was found to be less strenuous on the eyes and more comfortable for participants.

Visual stimulation was delivered through a set of MR-compatible binocular video goggles (NordicNeurolab Inc, Bergen, Norway) in blocks of 30 seconds followed by 1 minute of a white fixation cross on black background, repeated for 8 blocks, with 2 minutes of fixation cross presented before the first and after the final stimulus presentation.

3.4 MR-Imaging and Spectroscopy

All imaging and spectroscopy in this study was performed on a 3 T GE 750 Discovery Scanner from GE Healthcare (General Electric, Milwaukee, United States of America) using a standard 8-channel head coil from Invivo (Invivo corp., Gainsville, Florida, United States of America).

3.4.1 Article II Protocol

Following a 3-plane localiser sequence (2D Spin Echo, TE = 80 ms, FOV = 240 mm, slice thickness = 8 mm, slice spacing = 15 mm) a structural anatomical series was acquired using a 3D T1 weighted fast spoiled gradient sequence (FSPGR) (number of slices = 192, slice thickness = 1.0 mm, repetition time (TR) = 7.8 ms, echo time (TE) = 2.95 ms, field of view = 260 × 260 mm^2, flip angle = 14 degrees, matrix = 256 × 256). These structural images were used to position a 24 x 24 x 24 mm^3 voxel in the left pSTG, centred around the primary auditory cortex, aligned orthogonally in the axial scan plane with no angulation.

For the spectroscopy component of this study, a GABA specific MEGA-PRESS sequence (Mescher et al., 1998; Mescher et al., 1996) was used as it provides accurate and stable measurements of GABA, as well as a measurement of glutamate and glutamine combined as “Glx” (Henry et al., 2011). Spectra were acquired continuously using a MEGA-PRESS sequence (TE = 68 ms, TR = 1500 ms, 8-way phase cycling, editing at 1.9 and 7.5 ppm in alternating frames) for 628 paired repetitions, followed by 16 reference acquisitions without water suppression, for a total scan time of 31 minutes and 48 seconds, with tDCS initiated once ten minutes of spectroscopy had passed. Following stimulation, ~10 minutes of spectra were

acquired in the post-stimulation window in order to assess long term effects of stimulation.

This protocol was repeated twice for each participant, once with active and once with sham stimulation, counterbalanced for order, with both experimenters and participants blind to the stimulation condition.

3.4.2 Article III Protocol

As in article II, a 3-plane localiser sequence (2D Spin Echo, TE = 80 ms, FOV = 240 mm, slice thickness = 8 mm, slice spacing = 15 mm) was followed by a structural anatomical acquisition using a 3D T1 weighted fast spoiled gradient sequence (number of slices = 192, slice thickness = 1.0 mm, repetition time (TR) = 7.8 ms, echo time (TE) = 2.95 ms, field of view = 260 × 260 mm^2, flip angle = 14 degrees, matrix = 256 × 256) used to position a 31 x 26 x 24 mm^3 voxel in the medial occipital cortex, across the longitudinal fissure and angled parallel to the parieto-occipital sulcus.

In this experiment, both a standard short echo time PRESS sequence (TE = 35ms, TR = 1500 ms) comprising 620 frames for a total acquisition time of 15 minutes and 30 seconds and a GABA-specific MEGA-PRESS sequence (TE = 68 ms, TR = 1500 ms) of 310 paired acquisitions, also lasting 15 minutes and 30 seconds were used. Both sequences were performed consecutively and counterbalanced for order, with half of the participants having PRESS spectra acquired first and the other half MEGA-PRESS first. Both sequences were run with interleaved frames with and without water-suppression, providing an updated water reference from which BOLD effects could be derived.

Following spectroscopy, an fMRI acquisition was performed using an echo-planar imaging (EPI) sequence (TR = 3000 ms, TE = 30 ms, image matrix = 96 x 96, FOV = 220 mm, flip angle = 90°, slice thickness = 3.0 mm, slice spacing 0.5 mm).

3.5 Spectral Analyses

3.5.1 Time-Windowing and Block-Averaging Analysis

In article II a "windowing" method used by (Brix et al., 2017) for investigating within-session reproducibility of GABA measurements with MRS was adapted to perform time-resolved analyses of MR-spectra. In the original study, long acquisitions (20 minutes, 328 averages) were subdivided into smaller blocks to produce spectral series equivalent to shorter scan lengths, incorporating between 82 and 246 averages each. Quantitative analysis was performed on each block, then metabolite estimates compared with other blocks from the same acquisition in order to determine the effect of increasing the number of averages incorporated into a time-averaged spectrum on within-session reproducibility. In the present study, continuous acquisitions were subdivided into smaller blocks and used to construct a time course of metabolic changes, with the quantitative estimate of a metabolite at each time point being a weighted average of all metabolite estimates across all blocks of all sizes containing that time point.

Following pre-processing steps; phase adjustment, coil combination and spectral realignment, two separate spectral analyses were performed on the spectroscopy data acquired for article II, one using the adaptive, multi-resolution "time-windowing" approach, the other using a "block-averaging" approach.

There was some concern that active direct current in the scanner may affect spectral quality or produce spectral artefacts, and although none were observed during steady-state tDCS, artefacts were observed in spectral frames acquired during the ramping periods for both the active and sham stimulation conditions and were omitted from all subsequent analyses.

Quantification was performed with LCModel (version 6.3-1J) (Provencher, 1993, 2001) using a simulated basis set (Dydak et al., 2011) with Kaiser coupling constants (Kaiser, Young, Meyerhoff, Mueller, & Matson, 2008) to provide an estimate of average levels of GABA+MM (hereafter referred to simply as GABA), glutamate and

glutamine measured together as Glx, glutathione (GSH), NAA, and *N*-acetyl aspartate glutamate (NAAG). Metabolite levels were scaled relative to the unsuppressed water signal acquired at the end of each spectroscopy sequence.

For the time-windowing analysis, each continuous acquisition of 620 spectral frames, as 310 pairs of ON and OFF MEGA-PRESS spectra (see section 1.2.2), was taken and subdivided into contiguous blocks of different lengths and offsets relative to the start of the scan and subjected to quantification in LCModel. From this, a smoothed time course was constructed where for each time point in the curve, a quantitative estimate was made as an aggregate of all estimates from blocks containing that time point, weighted according to quality of fit (Figure 10).

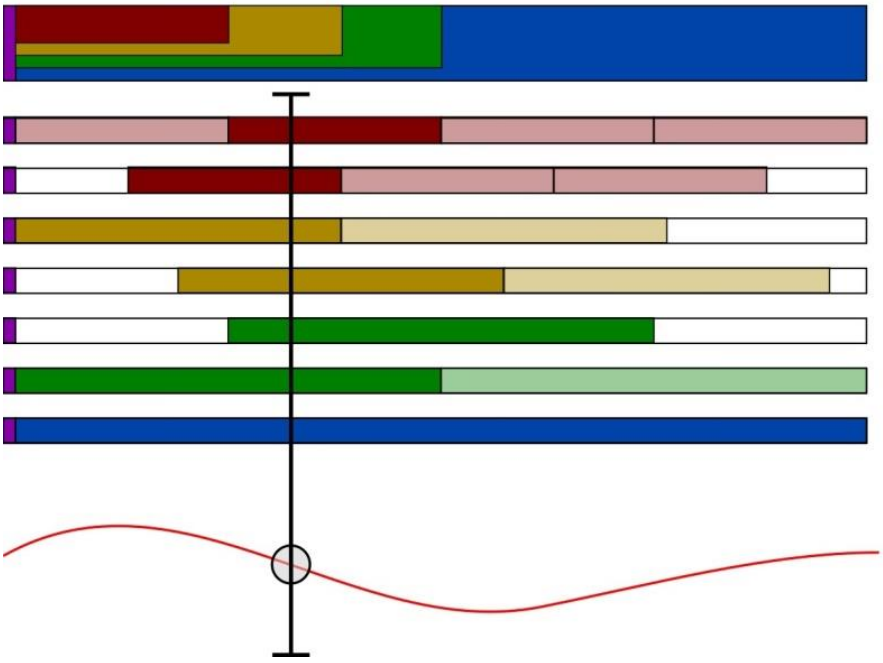

Figure 10 - Time windowing method schematic: The complete scan (blue) is subdivided into smaller blocks of different sizes (red, yellow and green) and different offsets relative to the beginning of the scan. A time course of the metabolite concentration is then produced (red curve) where the concentration at each time point is calculated as an average of all blocks containing that time point, weighted according to quality of fit.

Amid concerns that the time-windowing approach may have had a "smoothing" effect on the data, making small, acute changes difficult to detect, an alternate "block-averaging" analysis was performed. Two block-averaging analyses were performed for each participant, one with three time points and the other with five. Taking this approach, each continuous spectrum was first subdivided into three equal blocks of approximately 200 frames, not including those excluded due to spectral artefacts, comprising one pre-, during- and post-stimulation block. Each block was then subjected to quantitative analysis in LCModel to produce quantitative estimates at three time points. The during- and post- stimulation blocks were further subdivided into two blocks of approximately 100 frames each to produce a total of five time points: one pre-, two during- and two post-stimulation also analysed with LCModel.

3.5.2 BOLD-fMRI and Functional Spectroscopy

The purpose of the study presented in article III was to determine whether an MRS sequence could be used to measure both neural activity and metabolic changes by using the unsuppressed water signal as an indirect measurement of the BOLD-effect. Thus, the analysis of spectra acquired in this experiment may be divided into analysis of the BOLD-effect and analysis of biochemical changes.

For analysis of the BOLD-effect, FWHM values from the unsuppressed water signal were used to produce a time course of the average response for each 90 second stimulation block for each subject. The time course was constructed using a Gaussian-weighted combination of between 60 and 100 time points of the FWHM of the unsuppressed water signal to produce a smoothed curve, reflecting the change in FWHM of the unsuppressed water signal over the course of each 90 second stimulation block, including both active and rest periods.

Correlation analysis was performed between the average of all time courses for all participants and a model of the predicted haemodynamic response based on the canonical haemodynamic response function (HRF) from the Statistical Parametrical Mapping (SPM12) software toolkit.

For the analysis of biochemical changes, following phase adjustment, coil combination and realignment, spectral frames were separated into those acquired during stimulation blocks (active) and those collected before, between and after stimulation blocks (rest) to produce two time-averaged spectra per acquisition; one active and one rest spectrum. Thus, for each subject, four spectra were created, one active and one rest spectrum for the PRESS sequence and one active and one rest spectrum for the MEGA-PRESS sequence. Quantitative analysis was then performed for each spectrum using LCModel using a simulated basis set (Dydak et al., 2011) with Kaiser coupling constants (Kaiser et al., 2008).

Due to aberrant and inconsistent results in quantitative analyses of the PRESS spectra, they were excluded from subsequent analyses and the results from this study focus solely on spectra acquired using the MEGA-PRESS sequence and quantitative measures of glx, GABA and NAA from the difference edited spectrum and glx, NAA, choline, creatine, lactate and glucose from the OFF spectrum.

For analysis of the BOLD-fMRI data, all fMRI volumes acquired using the EPI sequence were first converted from DICOM to NIfTI format using the dcm2nii program (http://people.cas.sc.edu/rorden/mricron/dcm2nii.html). Preprocessing of the converted images was performed using the Matlab/SPM based toolbox CONN (Whitfield-Gabrieli & Nieto-Castanon, 2012). Volumes were realigned to the first volume in each set and unwarped to correct for subject motion (Friston et al., 1995) then spatially normalised to an EPI template based on the Montreal Neurological Institute (MNI) standard reference brain (Evans et al., 1992). Images were finally smoothed through spatial convolution with a 5 mm Gaussian kernel. Following pre-processing, beta values were extracted from a region of interest defined by a mask based on the placement of the spectroscopy voxel for each participant, using an in-house script drawing on the tools for NIfTI and ANALYZE image toolbox (https://se.mathworks.com/matlabcentral/fileexchange/8797-tools-for-nifti-and-analyze-image). For each participant three measurements were calculated from the BOLD signal for the whole acquisition: the average signal intensity during active blocks, rest blocks and the difference between active and rest blocks.

3.6 Statistical Analyses

All statistical analyses for articles II and III were performed using R (R Development Core Team, 2016).

In article II, for the 3- and 5-point analyses, a linear mixed effects model analysis was performed using the nlme package (Pinheiro, Bates, DebRoy, Sarkar, & Team, 2016) to examine the effect of tDCS on the concentrations of NAA, Glx and GABA, over time. The model specified the two groups of participants (active-first and sham-first) and time period as fixed effects, as well as an interaction effect between the two, and the subject as a random effect. This model was also used to investigate crossover effects between the active and sham stimulation conditions due to the within-subject design of the study, to determine whether order of stimulation, active first or sham first, may have had any significant effect on results and whether the stimulation condition in the first session had any lasting effect on the second.

In article III, a repeated measures t-test was performed on the metabolite data, comparing LCModel estimates for all measured metabolites between the rest and active conditions for each participant. For analyses of relationships between measurements of the BOLD effect and metabolic changes between the active and rest blocks, a correlation analysis was performed using the differences in all measured metabolite levels and BOLD signal changes as measured with fMRI between the active and rest blocks.

4. Results

4.1 Article I

The final review covered 41 articles in four categories: NAA (9 articles), Glutamate and Glutamine (23 articles), GABA (7 articles) and GSH (2 articles).

Irrespective of differences in acquisition parameters, experimental methodologies or heterogeneity amongst patient groups between studies, lower levels of NAA were consistently measured in schizophrenia patients compared to control subjects. However, there is evidence to suggest that changes in cellular environment may affect the T_2 times of NAA, more so than other MRS visible metabolites, and that the TE of pulse sequence used should be taken into consideration when interpreting results, as it may bias quantitative estimates. Additionally, given the role of *N*-acetylaspartylglutamate (NAAG) in regulating glutamatergic and GABAergic transmission, and the implication of these pathways in the pathophysiology of schizophrenia, future studies may benefit from investigating whether changes in NAA as measured with MRS are being driven by changes in the NAAG component of the measured signal by utilising sequences that may distinguish NAA from NAAG.

A large number of articles reviewed found reductions in either glutamate or glx, depending on the experimental design, in most regions of interest measured with the exception of the frontal lobe, where glx levels were found to be increased in patients with frequent and severe AVHs. One of the problems with reporting results relating to changes in the glx signal is that changes may be incorrectly attributed to changes in the glutamate component of the glx signal, or alternatively, covarying changes in glutamate and glutamine in opposite directions may not be detected as a change in the composite signal. Distinguishing glutamate from glutamine is difficult, particularly at field strengths of 3 T and less, but possible with the use of specialised sequences, many of which have been suggested, but there is little consensus regarding which may be the optimal method. Studies involving schizophrenia patients using sequences

that did distinguish between glutamate and glutamine often found increases in glutamine, or in the glutamine fraction of the glutamine/glutamate ratio, suggesting that increases in glx may be driven by increases in the glutamine fraction.

Unlike NAA, and glutamate and glutamine, no global increase or decrease in GABA levels were found in schizophrenia patients compared to controls. MEGA-PRESS remains the method of choice for detecting GABA levels at field strengths of 3 T and less, but it should be noted that unless otherwise specified, or that suppression techniques have been implemented, GABA levels as measured the MEGA-PRESS sequences and TE = 68 ms are unavoidably contaminated by an as yet unidentified macromolecule resonance at 1.7 ppm. Though changes in the signal may be attributed to changes in GABA, and changes are often in line with results predicted by the experimental hypothesis, it cannot by conclusively ruled out that changes are solely due to GABA and not the macromolecule. Several approaches to macromolecule suppression have been suggested, but where these have not been applied, results should ideally be reported with the caveat that the signals measured contain co-edited contributions from an unidentified macromolecule.

Like GABA, glutathione (GSH) may be measured with the MEGA-PRESS sequence at field strengths of 3 T or less, but there was no clear consensus regarding the optimal echo time for GSH (TE = 120 or 130 ms). The HERMES (Hadamard Encoding and Reconstruction of MEGA-Edited Spectroscopy) sequence may allow simultaneous editing of both GABA and GSH, allowing quantitative analyses of both to be performed.

4.2 Article II

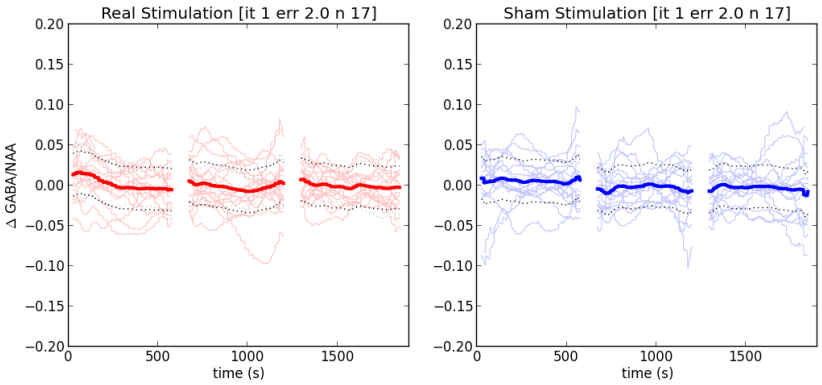

Figure 11 - GABA/NAA ratio as measured with time-windowing method

Preliminary analyses using the time windowing method (Figure 11) revealed no significant changes in GABA levels and were not subject to any further statistical analyses.

The linear mixed effects model on the 3- and 5-point analyses revealed no significant fluctuations in any of the metabolites of interest between any time points. Crossover analysis found no significant effects, indicating both that the order in which participants received the two different stimulation conditions had no significant effect on results and that there were no crossover effects from the first session significantly affecting the second. Finally, there was no significant difference in the change between groups over time, indicating no difference in fluctuations for any of the metabolite levels between active and sham conditions.

4.3 Article III

The fMRI data showed that the visual stimulation paradigm used induced neural activity within the region of interest with a mean difference in intensity between the active and rest blocks of 1.219 that was significant ($t(15) = 6.622$, $p < 0.001$, 95% CI =0.827, 1.612).

Linewidth changes in the unsuppressed water signal showed a very strong correlation with the predicted haemodynamic response function (r = -0.980, t(59) = -33.426, p < 0.001, 95% CI = -0.985, -0.958) with a change in mean FWHM between the active and rest blocks of 1.2% (Figure 12).

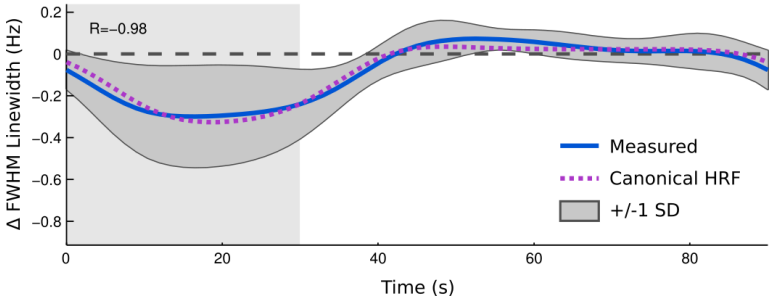

Figure 12 - Time resolved analysis of changes in FWHM of unsuppressed water signal compared to predicted haemodynamic response function (HRF) between active blocks (grey background) and rest blocks (white background) (from Article III).

Repeated measures t-tests showed no significant differences in GABA ($t(15) = -1.367$, p = 0.192, 95% CI = -0.157, 0.034) or Glx levels, whether measured from the difference spectra ($t(15) = -1.134$, p = 0.275, 95% CI = -0.376, 0.115) or the OFF spectra (t(16) = 0.007, p = 0.995, 95% CI = -0.376, 0.378) between the active and rest conditions, but that a small but very significant difference in creatine ($t(17) = 3.153$, p = 0.006, 95% CI = 0.031, 0.159) and choline (t(17) = 2.537, p = 0.021, 95% CI = 0.003, 0.038) was measured.

Correlation analysis on the difference in BOLD signal and metabolite levels between the active and rest conditions revealed a strong, significant correlation between Glx as measured from the off spectra and the BOLD signal (r = 0.661, $t(13) = 3.174$, p = 0.007, 95% CI = 0.225, 0.876) and a strong and significant negative correlation between Glx as measured from the off spectra with GABA measurements (r = -0.557, $t(13) = -2.420$, p = 0.031, 95% CI = -0.832, -0.063).

5. Discussion

The purpose of this thesis was to evaluate new approaches to MRS allowing improved temporal resolution and simultaneous measurement of neural activity and associated biochemical changes, with a specific focus on measuring Glx and GABA with the MEGA-PRESS sequence at 3 T, for application in the investigation of the pathophysiology of auditory-verbal hallucinations. Although no patients were involved, and this thesis was largely concerned with evaluating approaches to spectral analysis, the view to future applications in patients with auditory-verbal hallucinations influenced much of the experimental design and the work described in articles I and II.

Neither the time-windowing analysis, nor the 3- or 5-point analyses used in article II revealed any significant changes in any of the metabolites of interest in response to either active or sham stimulation. Considering the results, it was deemed inappropriate to include two analysis methods where neither showed a clear advantage over the other. Thus, the time-windowing method described in section 3.5.1 was omitted from the published article. It is important to note, however, that the omission of the results of the time-windowing approach do not represent a failure of the method, but rather that given the results of the experiment, it was more appropriate to present results from a more well-established approach than an experimental one. It is still believed that the time-windowing approach holds much potential, and that more experiments should be conducted to evaluate its performance.

The modified MEGA-PRESS sequence used in article III found no significant changes in Glx or GABA levels between the active and rest conditions, but did measure small but significant changes in creatine and choline. While this small but significant increase in two otherwise notoriously stable metabolites is most likely due to interference from the BOLD effect, the lack of any significant correlation between the differences in the BOLD signal and the differences in choline and creatine make the exact nature of these changes difficult to determine. Strong, significant

correlations were seen between Glx as measured from the OFF spectra and both the BOLD signal as measured with fMRI and with the GABA signal as measured from the difference edited spectra, consistent with previous findings that Glx levels appear to correlate positively (Apšvalka et al., 2015; Bednařík et al., 2015; Cleve et al., 2015; Gussew et al., 2010; Ip et al., 2017; Lin et al., 2012; Mekle et al., 2017) and GABA levels negatively (Bednařík et al., 2015; Chen et al., 2017; Cleve et al., 2015; Just & Sonnay, 2017; Lin et al., 2012; Mekle et al., 2017) with either neural activity or a task/stimulus positive condition. However, these correlations were only seen with Glx as measured from the OFF spectra and not the difference spectra. Both spectra have effectively measured the same metabolites at the same time, but the appearance of such a strong correlation using one method and not another raises questions as to which method may be more reliable, and whether the OFF spectra may be more sensitive to small changes in metabolite levels than the difference spectrum.

In a review of Ian Hacking's "Representing and intervening" (1983), Franklin (1984) raises the question "How do we validate observations that may only be made with one technique?" In his book, Hacking discusses how most modern scientific experiments depend on some sort of complex apparatus involved in the process of observation, e.g. an electron microscope or, in the case of this study, an NMR-spectrometer. Normally two different experimental procedures provide more evidence to support a hypothesis than repetitions of the experiment using the same procedure (Franklin & Howson, 1984). However, Franklin believes that reliance on extrapolation is "notoriously dangerous" and that a well corroborated theory of the apparatus is essential to validating findings made with the apparatus. Furthermore, one must be careful to distinguish between the theory of the apparatus and the theory of the phenomena, using the example that the use of a mercury thermometer in the investigation of whether an object's volume increases with temperature is objectionable, as the apparatus itself depends on the phenomenon under investigation.

Under this framework, the MR-scanner may be considered the apparatus and magnetic resonance the phenomenon under observation. The theory of magnetic

resonance and spectroscopy is will established, and has since its Nobel prize winning discovery in 1952 become a powerful tool in the field of analytical chemistry. The ability to use different kinds of apparatus to measure the same phenomenon presents one of the great problems in working with MRS, as there exists no practical alternative method for measuring dynamic changes in the metabolites of interest in the brain *in vivo*. Thus, evaluating the apparatus depends on the manipulation of two key parameters: The apparatus, namely the MR-scanner and the sequence parameters used, and the phenomenon, or the use of stimulation to manipulate levels of metabolites producing the resonance signals measured.

5.1 Limitations of MRS

5.1.1 Difference Spectra vs. OFF Spectra

Perhaps one of the greatest limitations to this project is that the experiments described in articles II and III present results obtained using the MEGA-PRESS sequence only. The ability to measure Glx and GABA simultaneously at 3 T made use of the MEGA-PRESS sequence both judicious and appropriate with respect to the aims of the project, but the results of the experiment performed in article III calls the reliability of quantitative estimates from the difference spectra into question.

Despite studies showing the MEGA-PRESS sequence to be comparable to standard short echo time PRESS sequences for quantitative estimates using the Glx signal (Henry et al., 2011; Nezhad et al., 2018) an investigation conducted by Maddock, Caton, and Ragland (2018) compared Glx as measured from the difference and OFF spectra using a GABA-optimised MEGA-PRESS sequence with measures of Glx using a glutamate-optimised PRESS sequence and found that estimates taken from the OFF spectra corresponded to PRESS values whereas those taken from the difference spectra did not. Furthermore, these discrepancies could not be attributed to factors such as frequency drift or differences in the basis sets used and the reason behind them remains somewhat unclear. The results presented in article III also found a moderate, but not significant correlation between GABA from the difference

spectrum and NAA from the OFF spectrum (r = 0.500, $t(12) = 2.000$, p = 0.069, 95% CI = -0.042, 0.815) but not with NAA from the difference spectrum suggesting that this phenomenon is not related to Glx specifically, but all measurements from the difference spectrum. However, as no reliable GABA measurement could be drawn from the OFF spectra, it remains unclear to what extent GABA signals in the difference spectra may be affected.

This finding raises two important issues regarding functional spectroscopy using MEGA-editing: Firstly, regarding reliability of quantitative estimates based on the difference spectra and secondly, where two methods for measuring the same phenomenon lack concordance, which may be considered more reliable without an alternative method for its measurement? The findings of Maddock et al. (2018) demonstrate only agreement between the OFF spectra Glx signals and glutamate optimised PRESS, but there is no way to determine which of the available methods may be more accurate when performing *in vivo* measurements. The correlations found with the OFF spectra Glx measurements are more consistent with previous findings, but again, this is largely a comparison between measurements taken from edited and unedited spectra across different pulse sequence parameters and scanner field strengths. The best evidence to suggest that the OFF spectra may be more reliable is the observation that the OFF spectra measurements of Glx showed a strong, positive correlation with the BOLD signal, consistent with current understanding of the physiological basis of the BOLD signal (Logothetis et al., 2001) however, for future applications of spectral editing in a functional capacity, this phenomenon warrants further investigation.

Alternatives for measuring GABA at 3 T without spectral editing do exist, such as the STEAM sequence (TE = 20 ms) as used by Kupers et al. (2009) and SPECIAL sequence (TE = 8.5 ms) used by Kühn et al. (2016), both of which were able to measure large, significant changes in GABA in the ACC, but neither of which is as well established or as well validated as the MEGA-PRESS technique.

5.1.2 Metabolite Quantification

As described in section 1.2.3, quantitative analysis of MR-Spectra is based, in principle, on the magnitude of a resonance signal relative to a reference signal, typically water or creatine. Although notorious for being a stable metabolite in the brain, reporting metabolite levels as a ratio relative to creatine may not be appropriate for certain applications as it has been shown to be affected by some pathologies and is susceptible to the influence of sex hormones (Hjelmervik et al., 2018). Comparison between quantitative measurements of GABA using MEGA-PRESS (TE = 68) was performed using both creatine at 24 independent sites (Mikkelsen et al., 2017) and water at 25 independent sites (Mikkelsen et al., 2019) finding comparable levels of variance in both measures. Currently, no one approach to quantification may be considered optimal, but rather appropriate to the experimental aims and design, and should be taken into consideration when interpreting results.

The issue of how to quantify metabolites is further complicated in functional spectroscopy studies, as it may become difficult to disentangle a true change in metabolite levels from changes in the estimated integrated area of the spectral peak due to BOLD interference.

In both experiments presented in this thesis, metabolite levels were quantified relative to an unsuppressed water signal acquired at the end of each spectral acquisition. Though this is common practice, the drawback to this approach in a functional paradigm is that comparing a dynamic signal to a stationary one means that two groups of signals, affected to different degrees by BOLD interference, are compared to a static, unaffected signal, increases the propensity for a “false positive” change to be detected. Conversely, using an internal reference such as creatine, it may difficult to determine a change if a change in the reference metabolite level has occurred which may lead to diminished or exacerbated differences in the apparent metabolite levels depending on the direction of the changes relative to the change in the reference. Given the susceptibility of both methods to error, metabolite levels were first calculated relative to the water signal acquired at the end of the acquisition as

this was this was the most commonly practiced method for MRS analysis and most prone to a “false positive” style of error. Should the results suggest interference or error, an alternative method would be investigated.

The lack of any significant changes measured in the tDCS experiment suggested no reason for alternative analysis. The small but significant changes in creatine and choline levels between active and rest conditions observed in the experiment presented in article III suggested possible BOLD interference, but the lack of correlation between the changes in creatine and the BOLD signal means that the nature of this change remains inconclusive. Furthermore, no significant changes were observed for NAA, also observed to exhibit similar stability (Stagg & Rothman, 2014) nor were any correlations observed between NAA and the BOLD signal. Though not presented in the article, or in this thesis, an analysis was performed using creatine as a reference, as it was believed that where both the creatine, Glx and GABA signals were subject to the same interference, it may help disentangle true changes in metabolite levels from BOLD interference. Analysis of metabolite levels measured relative to creatine revealed no significant changes in any of the metabolites of interest were measured between the active and rest conditions, including choline which had changed significantly when measured relative to water. Correlation analysis also revealed a strong, positive correlation between the Glx/Cr ratio and the BOLD signal ($r = 0.629$, $t(13) = 2.917$, $p = 0.012$, 95% CI = 0.172, 0.863) but also revealed a moderate, but not significant, correlation between Glx/Cr and GABA/Cr ($r = 0.400$, $t(13) = 1.575$, $p = 0.139$, 95% CI = -0.141, 0.757).

As mentioned in section 5, with no alternative measure to determine which of these two approaches may be more accurate, either method could have been presented and appropriately justified. Ultimately, the decision to present the results using the water reference was made based on water referencing being the preferred method amongst similar studies, allowing for more consistent comparison of results, and that the results from this approach were more consistent with previous findings, namely that changes in Glx correlated positively with the BOLD signal, and GABA negatively

with Glx. It should be noted that consistency is not equivalent to accuracy, and that all results should be interpreted in light of the quantification method used.

Another problem affecting metabolite quantification in the experiment presented in article II involves the experimental design. Participants were stimulated in blocks of 30 seconds with one minute of rest in between, with 2 minutes of rest before the first block and after the last. The effect of this is that the rest spectra have more spectral frames incorporated into the time-averaged spectra produced for each condition and subsequently, a higher SNR than the active spectra. The work of Brix et al. (2017) determined that for MEGA-PRESS (TE = 68 ms) in the OCC, the optimum scan length was 218 repetitions, or roughly 11 minutes with a repetition time of 1500 ms, above which inclusion of more spectral frames did not significantly improve SNR. Under each active visual stimulation block, with a repetition time of 1500 ms, 20 spectral frames were acquired. Every third of these was acquired without water suppression, leaving 14 frames per stimulation block and a total of 112 to contribute to the final active spectrum, equivalent to a total scan time of 2:48. It should be stressed that 218 was the optimal number, and that SNR improved significantly above 110 frames. Despite its apparent shortness, there was no evidence to suggest that the SNR of the active spectra were too low to achieve a reliable quantitative estimate, only that the SNR of the rest spectra were invariably higher.

5.2 Stimulation

As stated in section 3.3, the evaluation of the analysis methods presented in this thesis depended on some stimulus being able to induce a change that could be measured. It is believed that the absence of any significant changes measured between active or stimulus positive and rest or stimulus negative conditions may be due to the nature of the stimulus used rather than a fault of the analysis method.

5.2.1 Biochemical Changes and tDCS

tDCS was chosen as the stimulus for evaluating the time-windowing method in article II for two reasons: Firstly, previous studies showed it to have a measurable effect on GABA and Glx levels at the site of stimulation (Clark et al., 2011; Hone-Blanchet et al., 2016; Kim et al., 2014; Stagg et al., 2009) and secondly, it provided a way to reliably and consistently deliver the same stimulus to an area of choice in each participant. Given that the purpose of this project overall was to optimise a method for investigation into the pathophysiology of auditory verbal hallucinations, it was advantageous to have a stimulus that could directly facilitate biochemical changes in an area of interest, namely the pSTG.

The problem was that the changes in GABA and Glx levels found by other studies were found in other cortical areas, namely the motor cortex (Kim et al., 2014; Stagg et al., 2009), intraparietal (Clark et al., 2011) and prefrontal (Hone-Blanchet et al., 2016) cortices. The study presented in article II was, at the time of publication and to the best of the author's knowledge, the first to investigate biochemical changes in the pSTG. To reiterate the words of Franklin (1984) that reliance on extrapolation is "notoriously dangerous", it was assumed at the time that the effect of tDCS on GABA and Glx levels was global, and that active, anodal stimulation would increase Glx levels and decrease GABA levels but it should not have been taken for granted that all areas would respond in the same manner (Woods et al., 2016). The work of Westwood, Olson, Miall, Nappo, and Romani (2017) found that anodal tDCS in the temporal gyrus produced no change in performance on a range of reading and naming tasks, suggesting that the temporal gyrus may not be as susceptible to tDCS as other cortical areas. Thus, while the study presented in article II may prove useful for understanding of the effects of tDCS, the effectiveness of the time-windowing method remains inconclusive.

5.2.2 Visual Stimulation

One of the issues regarding the tDCS study in article II was that it was based on an assumption, that tDCS would elicit a biochemical response in the pSTG. For

evaluating the spectroscopy method described in article III, the experiment was designed to make as few assumption as possible, and to use a stimulus that had been demonstrated to unambiguously elicit neural activity and a biochemical response in an area of the brain where a spectroscopy voxel could be placed without concerns of being too close to bone, fluid or air or other elements that may cause spectral artefacts. The reasons for the red-black checkerboard design used in article III are outlined in section 3.3.2, and while this stimulus was appropriate for eliciting neural activity and a BOLD response, it may not have been the optimal method for eliciting an accompanying biochemical response.

Despite the intention to design an experiment based on others that had shown positive changes in both neural activity and Glx, glutamate or GABA levels, two key differences between the experiment presented in article III and the studies that influenced its design are that the majority of the studies stimulated participants in blocks of five minutes or longer, with the notable exception of Ip et al. (2017) where participants were stimulated in one minute blocks, and that a large number of these studies were performed at 7 T (Bednařík et al., 2015; Lin et al., 2012; Mangia et al., 2006; Mangia et al., 2007; Mekle et al., 2017; Schaller et al., 2013). Taken together, these differences suggest two possibilities: Firstly, that the SNR in the active spectra was too low to resolve changes in metabolite levels from noise, and that the changes in glutamate measured in studies where participants were stimulated for longer blocks ($\geq$ 5 mins) are more closely related to changes in energy metabolism than neurotransmission. Both are possible.

The mean change in glutamate in OCC in response to photic stimulation was found to be around 3% (Mullins, 2018). The intrinsically higher SNR at 7 T allows for higher sensitivity to small changes and this, coupled with the aforementioned loss of SNR due to the low number of frames incorporated into the active spectra, may suggest that SNR was simply too low to adequately quantify glx levels in the active conditions. However, a strong positive correlation was still able to be detected between Glx levels and the BOLD signal, and as mentioned previously, there was no

evidence based on the spectral quality metrics to suggest SNR was too low for adequate quantitative analysis.

Schaller et al. (2014) suggest that increases in glutamate and lactate as measured with MRS at 7 T are ubiquitous to increased neural activity in the brain, with the increase in lactate representing an upregulation of non-oxidative metabolism. Though not reported in article III, changes in lactate were also assessed, but not found to change significantly between the active and rest groups ($t(15) = 0.715$, $p = 0.486$, 95% CI = -0.082, 0.164). Though this observation suggests no measurable increase in non-oxidative metabolism in the region of interest, a BOLD effect was measured by both fMRI and the changes in the linewidth of the water signal. Taken together, this suggests that the experimental design stimulated the area of interest sufficiently to induce an increase in neural activity, but not enough to cause a shift in energy metabolism, making it ideal for disentangling changes in glutamate related to neurotransmission from those related to energy metabolism, but not for evaluating the spectroscopy method.

5.3 The Nature of Metabolic Changes

Beyond the duration of stimulation blocks and the SNR of acquired spectra, two more important factors that have been demonstrated to influence both the magnitude and nature of metabolic changes measured with functional MRS include how stimulation is presented, i.e. block- vs. event related design, and from where in the brain spectra are acquired.

The use of block designs in fMRI and, subsequently, fMRS experiments is largely influenced by its established use in PET experiments (Amaro & Barker, 2006). A comparison between block- and event related experimental designs in how glutamate or Glx levels change with stimulation found an astonishing difference of an average increase of 4.749% (±1.45%) for block designs and 13.429% (±3.59) in studies using event related paradigms (Mullins, 2018). Furthermore, studies measuring changes in the OCC in response to visual stimulation tend to show a much smaller response,

with a mean increase of glutamate of 2.318% (±1.227%) compared to painful stimuli, where the mean increase in glutamate was 14.458% (±3.736%). It should be noted that of the articles reviewed by Mullins (2018), none of those investigating responses to visual stimulation in the OCC used an event-related design, those using an event-related design in the lateral occipital cortex (LOC) measured changes in response to an object recognition task (Apšvalka et al., 2015; Lally et al., 2014) and those using an event-related design investigated changes in response to pain in the insula (Gussew et al., 2010) and anterior cingulate cortex (ACC) (Cleve et al., 2015).

Between the active blocks in the experiment in article III, participants were presented with a white fixation cross. The fMRI acquisition and the change in the linewidth of the unsuppressed water signal both show a change in neural activity, but the fact that at all times the participant was engaged in the act of some form of visual stimulation means that the primary visual processing areas in the OCC were likely engaged throughout the experiment, and that the change in activity measured is relative. The insula and ACC on the other hand are components of the extrinsic mode network (EMN) (Hugdahl, Raichle, Mitra, & Specht, 2015), a generalised network that is up-regulated in response to tasks that exceed a particular cognitive demand and/or require the processing of novel information, that has been defined as a complement to the default mode network (DMN), which is preferentially active when individuals are not focused on the external environment and does not include the insula or ACC (Buckner, Andrews-Hanna, & Schacter, 2008).

Taken together, the findings of Mullins (2018) and the experiments presented in this thesis suggest that independent of the stimulus presentation design or field strength of the scanner used, the larger changes in glutamate observed may not only reflect increases in activity, but perhaps the up-regulation of the EMN in response to increased cognitive load above a certain threshold.

6. Conclusion and Future Perspectives

This thesis presents new approaches to the use of MR-spectroscopy in the investigation of the pathophysiology of auditory-verbal hallucinations with two potentially useful methods for both the analysis of MR-spectra in a time-resolved manner, and the simultaneous measurement of neural activity and quantitative measurement of metabolic activity.

The findings of the review article suggest that further investigation into the pathophysiology of schizophrenia may benefit from the use of sequences allowing the discrimination of metabolites often measured together as a composite signal, such as the distinction between NAA and NAAG and between glutamate and glutamine instead of the Glx signal.

While the MEGA-editing method remains the method of choice for the detection of GABA, even at field strengths higher than 3 T, the findings presented in article III suggest that difference edited spectra may not be as reliable nor as sensitive to small changes in metabolite levels as unedited spectra. Options for quantitative analyses of GABA without the use of spectral editing warrant further investigation.

Finally, future studies evaluating methods for functional or time-resolved spectroscopy would benefit from experimental designs used to elicit the largest biochemical response possible before further refinement. Ideal approaches suggest acquiring spectra from the ACC or insula, or other region associated with the EMN coupled with either a painful stimulus, or one shown to cross the threshold of cognitive demand for up-regulation of the EMN.

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No Effects of Anodal tDCS on Local GABA and Glx Levels in the Left Posterior Superior Temporal Gyrus

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A number of studies investigating the biological effects of transcranial direct current stimulation (tDCS) using magnetic resonance spectroscopy (MRS) have found that it may affect local levels of γ-aminobutyric acid (GABA), glutamate and glutamine (commonly measured together as "Glx" in spectroscopy), and N-acetyl aspartate (NAA), however, these effects depend largely on the stimulation parameters used and the cortical area targeted. Given that different cortical areas may respond to stimulation in different ways, the purpose of this experiment was to assess the as yet unexplored biological effects of tDCS in the posterior superior temporal gyrus (pSTG), an area that has attracted some attention as a potential target for the treatment of auditory verbal hallucinations in schizophrenia patients. Biochemical changes were monitored using continuous, online MRS at a field strength of 3 Tesla. Performing intrascanner stimulation, with continuous spectroscopy before, during and after stimulation, permitted the assessment of acute effects of tDCS that would otherwise be lost when simply comparing pre- and post-stimulation differences. Twenty healthy participants underwent a repeated-measures experiment in which they received both active anodal and sham intrascanner stimulation in a stratified, randomized, double-blind experiment. No significant changes in GABA, Glx, or NAA levels were observed as a result of anodal stimulation, or between active and sham stimulation, suggesting that a single session of anodal tDCS to the pSTG may be less effective than in other cortical areas that have been similarly investigated.

Keywords: tDCS, GABA, Glultamate, magnetic resonance spectroscopy, MRS

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INTRODUCTION

Transcranial direct current stimulation (tDCS) is a non-invasive neurostimulation technique that uses constant, low level (0.5–2.0 mA) direct current to modulate cortical excitability in a polarity dependent manner (1). Nitsche and Paulus (2) used the magnitude of motor-evoked potentials (MEP) as generated by transcranial magnetic stimulation (TMS) as an indication of changes in excitability and found that tDCS was able to induce changes in excitability of up to 40%, with anodal stimulation having an excitatory effect, and cathodal stimulation having an inhibitory

effect. Subsequent studies showed that effects may outlast the duration of stimulation, with short applications inducing excitability shifts during stimulation, and ∼10 min or more of stimulation producing persistant effects lasting up to 90 min after current flow has ceased (3) suggesting that tDCS has the ability to induce long term potentiation (LTP)-like effects on synaptic plasticity (4).

Due to its purported effects on excitability and synaptic plasticity, tDCS has been investigated as a potential treatment for a range of neurological and psychiatric disorders such as Parkinson's disease (5), depression (6), and for the treatment of auditory verbal hallucinations in schziophrenia. A case reported by Homan et al. (7) found that cathodal tDCS halfway between T3 and P3 in the 10–20 electroencephelography (EEG) system was successful in alleviating both hallucinations (−60% Hallucination Change Scale (HCS) score) and global symptoms (−20% Postive and Negative Syndrome Scale (PANSS) score). A randomized control trial conducted by Brunelin et al. (8) using a similar stimulation paradigm at 2.0 mA also showed improvement in hallucinations (−31% Auditory Hallucination Rating Scale (AHRS) score) and global symptoms (−13% PANSS score). Subsequent studies, using similar stimulation parameters have found both reductions (9) and no significant differences (10, 11) in symptoms. While tDCS shows great promise as a potential treatment for schizophrenia, the lack of consistent findings between these studies highlight the need for a deeper understanding of the effects of tDCS.

Although generally accepted that anodal stimulation typically facilitates excitability and cathodal stimulation inhibits excitability (12), studies have shown that the effects of tDCS on excitability are not so simplistic, and depend on a number of factors such as electrode size and placement, stimulation intensity and duration, as well as the orientation of neurons relative to the stimulating electrodes (12–14). Furthermore, Batsikadze et al. (15) found that while 20 min of cathodal stimulation at 1.0 mA had an inhibitory effect, 20 min of cathodal stimulation at 2.0 mA had an excitatory effect, increasing the magnitude of measured MEPs. Esmaeilpour et al. (16) showed that the dose-response relationship in tDCS is not necessarily linear, and that although increasing current produces a corresponding increase in brain electric field, it may not necessarily enhance a neurophysiological, behavioral or clinical outcome. As Woods et al. (14) caution, it cannot be taken for granted that what is effective in a particular cortical area is transferable and applicable to others, rather recommending a "titration" of parameters.

Abbreviations: BOLD, Blood Oxygen Level Dependent; EEG, Electroencephalography; ERETIC, Electronic reference to access *in vivo* concentrations; FWHM, Full width at half maximum; GABA, Gamma-aminobutyric acid; Glx, Glutamate and glutamine; GSH, Glutathione; LTP, Long-term potentiation; MEGA-PRESS, Mescher-Garwood point resolved spectroscopy; MEP, Motor evoked potential; MRS, Magnetic Resonance Spectroscopy; NAA, N-acetylaspartate; NAAG, N-acetylaspartylglutamate; pSTG, Posterior superior temporal gyrus; tDCS, Transcranial direct current stimulation; TE, Echo time; TMS, Transcranial Magnetic Stimulation; TR, Repetition time

Despite the observed effectiveness of tDCS, the exact mechanisms by which it works are not yet fully understood. Horvath et al. (17) show that changes in cognitive effects alone may be an unreliable measure of effectiveness. Computational forward models and simulations have been useful in imaging current flow, aiding in the design of stimulation paradigms (18) but do not provide information about neuronal responses to delivered current or whether the effect is excitatory or inhibitory in nature.

Krause et al. (19) suggest that tDCS may modulate the excitation/inhibition balance, that is, the relative contributions of excitatory and inhibitory inputs to a neural circuit corresponding to a neuronal event. Using *in vivo* magnetic resonance spectroscopy (MRS), the excitation-inhibition balance may be characterized in terms of the local concentrations of the excitatory neurotransmitter glutamate and inhibitory neurotransmitter gamma-aminobutyric acid (GABA). Studies that have used MRS to investigate the effect of tDCS have found anodal tDCS to reduce local cortical GABA concentration in the motor cortex (20, 21) and to increase local concentrations of glutamate and glutamine, measured together as "Glx," and N-acetyl aspartate (NAA) in the intraparietal and prefrontal cortices (22, 23), the observed reduction in inhibitory neurotransmitter levels and concurrent increases in excitatory neurotransmitter levels being consistent with the facilitatory nature of anodal stimulation. Thus, *in vivo* MRS provides a window into the biochemical events underlying tDCS that may also be used as a biomarker indicating the effectiveness and nature of a stimulation paradigm.

In this study, MRS was used to investigate the acute biochemical effects of tDCS in validating its potential for use as a treatment for auditory-verbal hallucinations in schizophrenia. However, rather than simply comparing pre- and post-stimulation spectral acquisitions, biochemical changes were measured continuously using online MRS in a manner similar to those used by Bachtiar et al. (24) and Hone-Blanchet et al. (23). By acquiring spectra continuously over the course of stimulation, spectral frames could be combined in such a way that metabolite levels could be measured and tracked before, during and post stimulation, allowing better insight into the acute effects of stimulation as opposed to the lasting effects. Findings in other cortical areas suggest that if anodal tDCS were to have a similar effect on the local excitation-inhibition balance, it may be measured as a statistically significant increase in Glx and NAA levels (22, 25) and decrease in GABA levels (20, 21, 24) and that these changes would be significantly different under active stimulation when compared to sham.

MATERIALS AND METHODS

This study was carried out in accordance with the recommendations and ethical approval of the regional committee for medical and health research ethics (REK-Vest) REK case number 2013/2342. All subjects gave written informed consent in accordance with the Declaration of Helsinki and the

guidelines drawn up by The Norwegian National Research Ethics Committee for medical and health research (NEM).

Participants

Twenty healthy participants (mean age: 25 years, range: 19–32; 10 male) participated in the study. All participants were required to complete a Norwegian language version of the Edinburgh handedness inventory (26) to determine right-handedness in an attempt to control for issues related to lateralization of cortical areas, such that stimulation in the left hemisphere affects approximately the same functional area in each participant. The test assessed dominance of right and left hand in performing 10 everyday activities to produce a score ranging between −100 (exclusively left handed) and +100 (exclusively right handed), participants with a score greater than +40 were considered to be right handed and were permitted into the study (mean score: +80, SD: 24). Based on self-report, participants were free from psychiatric and neurologic conditions and had not used any psychoactive/psychotropic substances, including no smoking or other tobacco based or nicotine containing products, for 6 months prior to participating in the experiment. Participants were also instructed not to consume alcohol for at least 24 h prior to participation.

Data from one female participant was omitted from final analyses due to abnormally high measurements of Glx more than three standard deviations above the group mean (Glx levels almost 5 times higher than average values), suggesting an error in spectral acquisition.

tDCS Stimulation

Stimulation was performed using an MR-compatible DC-Stimulator MR (neuroConn GmbH, Ilmenau, Germany) fitted with two 5 × 7 cm (35 cm^2) MR compatible rubber electrodes. Given that the motivation for this study was the potential for tDCS to be used as a treatment for schizophrenia, stimulation parameters were chosen to emulate those used in previous studies. Intensity was set at 2.0 mA (27) and although the majority of studies using tDCS as a treatment for auditory-verbal hallucinations have stimulated for 20 min, the prohibitively long scan time this would necessitate in order to have three equally long spectroscopy windows meant that stimulation had to be limited to 10 min. The anodal electrode was placed with the center of the pad on an area over the pSTG, such that the lower corners of the 7 cm edge of the electrode touch points T3 and T5 in the EEG 10-20 system. The cathodal electrode was placed over the contralateral orbitofrontal cortex, a site commonly used in tDCS montages for placement of the reference electrode (2, 12) such that the center of the electrode covered point AF8 in the EEG 10–20 system. Each electrode was coated with a layer of Ten20 conductive paste (Weaver and Company, Aurora, United States of America) at the interface between electrode and skin to improve both adhesion and conductivity. Once the electrodes were in place, participants were placed in the scanner with electrodes attached but not connected to the stimulation box. Electrodes were only connected prior to spectroscopy sequences.

This study followed a stratified, randomized, double-blind design, with both participants and experimenters blind to the stimulation condition. Each subject participated in two MR-scanning sessions with tDCS: one with active and one with sham stimulation, separated by a wash-out period of 1 h outside of the scanner (12, 28) counterbalanced for order. Double-blinding was performed by having the stimulation condition determined by a code, independently predetermined by a researcher not present at the stimulation, such that each participant underwent both active and sham stimulation conditions and that equal numbers experienced active and sham stimulation as the first condition.

MR-Imaging and Spectroscopy

All imaging and spectroscopy was performed on a 3 T GE 750 Discovery Scanner from GE Healthcare (General Electric, Milwaukee, United States of America) using a standard 8-channel head coil from Invivo (Invivo corp., Gainsville, Florida, United States of America).

Following a 3-plane localizer sequence (2D Spin Echo, TE = 80 ms, FOV = 240 mm, slice thickness = 8 mm, slice spacing = 15 mm) structural anatomical imaging was performed using a 3D T1 weighted fast spoiled gradient sequence (FSPGR) (number of slices = 192, slice thickness = 1.0 mm, repetition time (TR) = 7.8 ms, echo time (TE) = 2.95 ms, field of view = 260 × 260 mm^2, flip angle = 14 degrees, matrix = 256 × 256). These structural images were used to position a 24 × 24 × 24 mm^3 voxel for the spectroscopy component of this experiment in the left pSTG, centered around the primary auditory cortex, aligned orthogonally in the axial scan plane with no angulation (**Figure 1**).

Since the aim of this study was to characterize acute biochemical changes in terms of the excitation-inhibition balance, a GABA specific MEGA-PRESS sequence (29) was used as it provides accurate and stable measurements of GABA, as well as a measurement of glutamate and glutamine combined as "Glx" (30). Spectroscopy was performed using a MEGA-PRESS sequence (TE = 68 ms, TR = 1,500 ms, 8-way phase cycling, editing at 1.9 and 7.5 ppm in alternating frames) of 628 paired repetitions, followed by 16 unsuppressed reference acquisitions for a total scan time of 31 min and 48 s. Once 10 min of spectroscopy had elapsed, stimulation was initiated at the control box located outside the scanner at the control room. Active stimulation was delivered for 10 min with 24 s of ramping time both before and after the stimulation/sham period at a constant intensity of 2.0 mA. For the sham stimulation condition, intensity was ramped up to 2.0 mA over 24 s, then delivered for another 40 s, before being ramped down to zero, giving participants a similar sensation to that they would experience during active stimulation. Spectroscopy acquisition continued for 10 min in order to assess post-stimulation effects (**Figure 2**).

Spectral Analysis

While no spectral artifacts were observed during steady-state tDCS stimulation, mild artifacts were seen in spectral frames acquired during the ramping periods for both active and sham stimulation. Frames from these periods were omitted from all subsequent analyses.

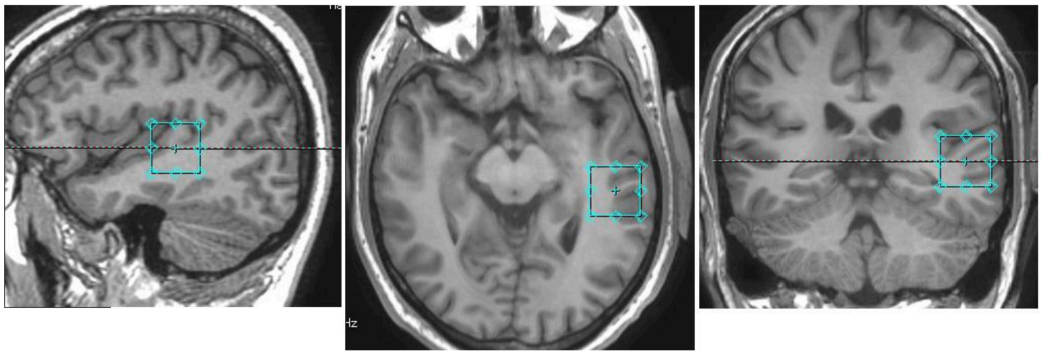

FIGURE 1 | Voxel placement in the pSTG in one participant: Sagittal (left), axial (middle), and coronal (right) views overlayed on an anatomical scan.

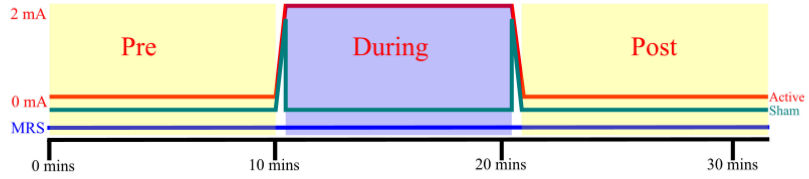

FIGURE 2 | tDCS and MRS: Each participant received both active and sham stimulation, separated by a washout period of 1 h, counterbalanced for order. 24 s of ramping up and down were incorporated into both active and sham stimulation. MRS was acquired constantly throughout each session. The pre-, during, and post-stimulation spectroscopy windows did not include frames acquired during ramping.

Following phase adjustment, coil combination, and realignment, each continuous acquisition was first subdivided into three smaller blocks of ∼10 min, with exact length depending on how many frames were excluded due to ramping artifacts, comprising a pre-, during-, and post stimulation block for each session, hereafter referred to as a three point analysis. Frames within each block were then averaged together and within each block, ON, and OFF spectrum pairs were subtracted to produce a difference spectrum then subjected to quantitative analysis with LCModel (version 6.3-1J) (31, 32) using a simulated basis set (33) with Kaiser coupling constants (34) to provide an estimate of average levels of GABA, glutamate and glutamine measured together as Glx, glutathione (GSH), NAA, and N-acetyl aspartate glutamate (NAAG). Metabolite levels were scaled relative to the unsuppressed water signal acquired at the end of each spectroscopy sequence.

One issue that affects MEGA-edited GABA spectroscopy is co-editing of macromolecule (MM) resonances at 1.7 ppm contaminating the GABA signal in the difference spectrum. GABA, in this report, refers to both GABA and the co-edited macromolecule, typically denoted GABA+ (35).

To further investigate acute effects of tDCS, and eliminate the possibility short-lived metabolic fluctuations being obscured through averaging, a second analysis was performed in which the during- and post-stimulation blocks were further subdivided into two smaller windows in an attempt to uncover any changes in metabolite concentration during this period, thus providing five time points over the acquisition, hereafter referred to as the five point analysis: one 10 min pre-stimulation window, two 5 min during-, and two 5 min post-stimulation windows.

MRS signals have been demonstrated to be susceptible to line-broadening artifacts associated with local blood-oxygen-level dependent (BOLD) effects (36). As an indication of potential BOLD interference, the full width at half maximum (FWHM) values as determined by LCModel were used as a measure of quality control, to ensure the MRS signal had not been significantly affected between time points.

Statistical Analysis

Statistical analyses were performed using R (37) and the nlme package (38) to perform a linear mixed effects model analysis of the effect of tDCS on the concentrations of three metabolites of interest, namely NAA, Glx, and GABA, over time. This model specified two groups of participants (active-first and sham-first) and time period as fixed effects as well as an interaction effect between the two, with the subject as a random effect. This model was also used to investigate crossover effects between the active and sham stimulation conditions due to the within-subject design of the study, to determine whether order of stimulation, active first or sham first, may have had

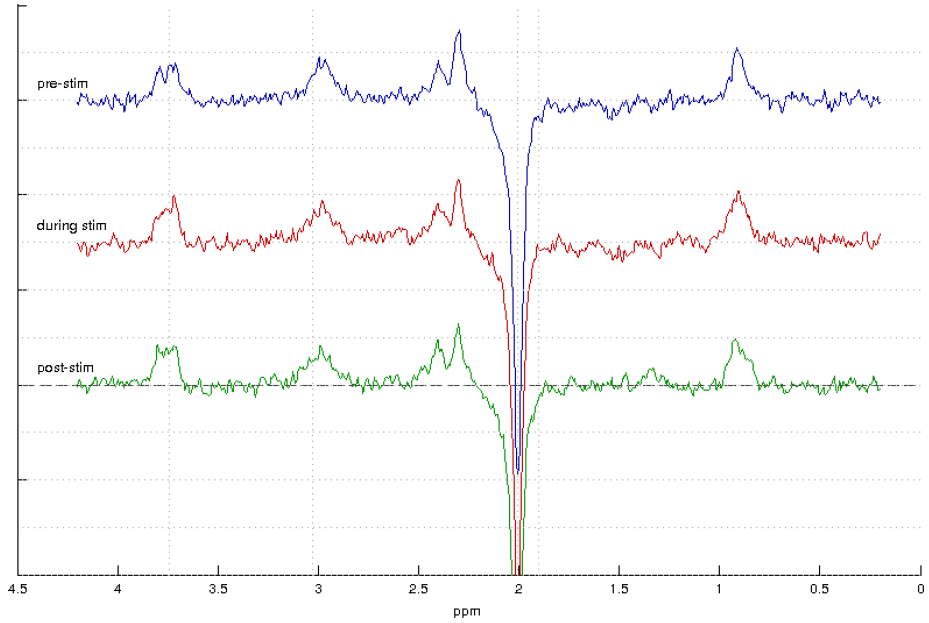

FIGURE 3 | MEGA-PRESS spectra from one participant showing spectra acquired during the pre-stimulation window (top, blue) during stimulation (middle, red) and post-stimulation (bottom, green).

any significant effect on results and whether the stimulation condition in the first session had any lasting effect on the second. The same model was used for both the 3-point and 5-point analyses.

RESULTS

Sample spectra from the three-point analysis of an individual participant are shown in **Figure 3** along with spectral quality metrics for all participants in **Table 1**. A linear mixed effects model of the average metabolite concentration across three time windows (pre-, during-, and post-stimulation) revealed no significant fluctuations in any of the metabolites of interest between any time points (**Figure 4** and **Appendix A**). Similarly, no significant fluctuations in any of the metabolites of interest were found between any time points in the five-time point analysis (**Figure 4** and **Appendix B**).

No significant crossover effects were found (**Figure 4**, **Appendices A, B**) indicating both that the order in which participants received the two different stimulation conditions had no significant effect on results and that there were no crossover effects from the first session significantly affecting the second. There was no significant difference in the change between groups over time, indicating no difference in fluctuations for any of the metabolite levels between active and sham conditions.

The FWHM as reported by LCModel was used as an indication of potential BOLD interference (**Tables 2**, **3**), but saw very little fluctuation between time points, making BOLD interference an unlikely source of error.

DISCUSSION

The montage and stimulation parameters used in this experiment did not induce a statistically significant effect on Glx, GABA, or NAA levels as measured with the MRS sequence used, and there was no significant difference in response observed between the active and sham stimulation conditions.

The active hypothesis for this experiment was informed by previous studies in which active anodal stimulation was found to be associated with increases in Glx and NAA levels (22, 23) and decreases in GABA levels (20, 21, 24) as measured by MRS. In comparing these studies with the findings presented here, there are three key elements to be considered, namely the stimulation parameters, the MRS acquisition parameters and the site of stimulation and spectroscopy.

As stated in section tDCS Stimulation, due to limitations of the experimental design, stimulation could only be delivered for 10 min as opposed to the 20 min previously used in the treatment of schizophrenia symptoms. Although as little as 7 min of stimulation has been

shown to induce lasting effects after stimulation has ceased (39), it cannot be taken for granted that the 10 min delivered in this session was sufficient to induce a change. While the stimulation window was shorter than the 30 min used by both Clark et al. (22) and Hone-Blanchet et al. (23) and the 20 min and 15 min used by Bachtiar et al. (24) and Kim et al. (20), respectively, Stagg et al. (21) were able to detect significant changes in GABA and Glx levels in the left sensorimotor cortex using a similar

TABLE 1 | Spectral Quality: FWHM, SNR, and mean %CRLB for GABA and Glx for each stimulation window.

Window	FWHM (Hz)	SNR	Mean GABA %CRLB	Mean Glx %CRLB
Pre Stim	8.22 ± 2.34	20.47 ± 4.71	5.12	4.54
During Stim	8.39 ± 2.41	20.83 ± 4.20	5.16	4.41
Post Stim	8.03 ± 2.18	21.67 ± 4.04	4.91	4.24

TABLE 2 | Average FWHM and standard deviation (sd) as estimated by LCModel for the 3-point analysis.

	Mean FWHM—3-point analysis (Hz)					
	Pre	sd	During	sd	Post	sd
Active	7.95	1.86	7.98	1.79	7.59	1.38
Sham	8.46	2.24	8.64	2.24	8.32	2.30

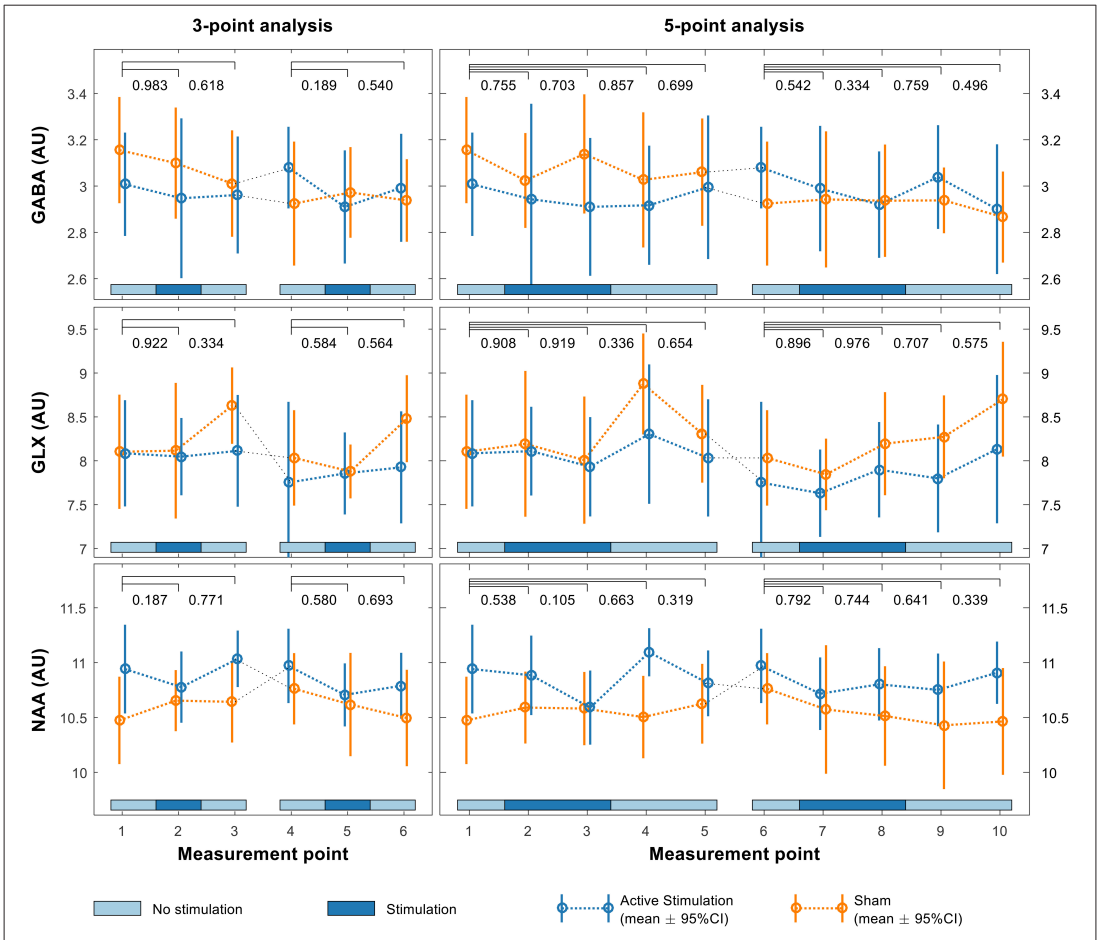

FIGURE 4 | Linear mixed effects model of GABA (upper row), Glx (middle row) and NAA (lower row) levels in the pSTG for both the first and second sessions using both 3-point (left) and 5-point (right) analyses. *p*-values shown above each plot indicate significance of interaction effects at each time point relative to baseline.

TABLE 3 | Average FWHM and standard deviation (sd) as estimated by LCModel for the 5-point analysis.

	Mean FWHM−5-point analysis (Hz)									
	Pre	**sd**	**During1**	**sd**	**During2**	**sd**	**Post1**	**Sd**	**Post2**	**sd**
Active	7.95	1.86	8.00	1.77	7.94	1.69	7.74	1.73	7.77	1.55
Sham	8.46	2.24	8.50	0.02	8.46	2.06	8.28	2.42	8.46	1.96

MEGA-PRESS sequence at 3 T given only 10 min of anodal stimulation at 1.0 mA. The findings of Batsikadze et al. (15) and Esmaeilpour et al. (16) suggest it is possible that stimulating at 2.0 mA had a different effect to the one predicted. However, studies conducted by Brunelin et al. (8) and Mondino et al. (9) both found significant reductions in symptoms of auditory-verbal hallucinations using stimulation in this area following cathodal stimulation at 2.0 mA, suggesting an issue more likely related to electrode polarity than stimulation intensity. While no significant changes, nor non-significant tendencies toward changes in any of the metabolites under investigation were seen during stimulation, even in the five-point analysis, it is unlikely that allowing a full 20 min of stimulation would induce a measureable effect, though it cannot be ruled out conclusively.

One of the unique features of this study was the use of continuous, online MRS as opposed to separate acquisitions. While Hone-Blanchet et al. (23) also acquired spectra during stimulation, also using a MEGA-PRESS sequence with an echo time of 68 ms and 11 min acquisition blocks, their study does not include a pre-stimulation window. Similarly, Clark et al. (22) acquired multiple spectra during the pre- and post-stimulation windows, also using a MEGA-PRESS sequence with an echo time of 68 ms, but with spectra acquired sequentially rather than continuously in blocks of 4 min and 48 s. While there is little difference in terms of the resultant spectra whether acquired continuously or sequentially, acquiring separate scans may introduce more variability as each pre-scan affects parameters such as shim, gain adjustment and center-frequency tuning between each segment. It may be considered more robust to acquire all spectra with the same parameters, as was done in this study with single continuous acquisitions. Compared with previous studies using similar sequences, comparable or shorter acquisition times, and smaller voxel sizes, i.e., $20 \times 20 \times 20$ mm^3 (21, 22, 24), there is little evidence to suggest an error in the MRS acquisition. Intuitively, a larger voxel size provides a higher signal-to-noise ratio, but may come at the expense of some focality in terms of covering the site of stimulation. It is possible that the larger voxel size used in this study may have incorporated spectra from cells not affected by stimulation. However, the voxel dimensions are still small compared to the surface area of the stimulating electrode, and tDCS is not a particularly focused stimulation technique.

The most significant difference between this study and other studies that have measured biochemical changes associated with tDCS with MRS is the cortical region being investigated, both as a stimulation site and volume of interest in spectroscopy. As Woods et al. (14) illustrated, it cannot be taken for granted that all cortical areas will respond to stimulation in the same manner, and compared to areas such as the sensorimotor cortex and frontal areas such as the dorsolateral prefrontal cortex, the temporoparietal and temporal regions have not been quite as thoroughly investigated. One study investigating the use of anodal tDCS in an adjacent cortical area, namely the left mid-posterior temporal gyrus, on improving performance in a range or reading and naming tasks (40) did not find any significant improvement in performance. Although different stimulation parameters were used, the agreement between the null-findings of this and the present study suggest it is possible that the pSTG and adjacent areas in the region, are not as responsive to anodal stimulation as other areas that have been investigated, but that the effectiveness of tDCS as a treatment for hallucinations is based on its ability to modulate over-active areas in the brain with cathodal stimulation. That is to say, anodal stimulation may not affect excitability in the pSTG, but cathodal stimulation may be effective in modulating activity in over-active or pathologically active networks such as those that might be associated with hallucinations. Computer modeling may be able to determine whether the responsiveness of this cortical area may be due to anatomical features such as skull thickness or cerebrospinal fluid density. It may be of interest to repeat a similar experiment looking at the effects of cathodal stimulation in this area in conjunction with computer models that may be able to determine whether the absence of an observed affect may be attributed to issues of anatomy and current flow.

In an investigation into the effect of active, intrascanner tDCS on the BOLD response as measured with functional MRI, Antal et al. (41) found that the presence of an electric current in the magnetic field inside an MRI scanner produces artifacts that may result in confounding false-positive activity patterns. While mild artifacts were observed during the ramping periods before and after stimulation, and these spectral frames were removed from subsequent analyses, there were no artifacts observed during active or sham stimulation periods. Furthermore, there were no statistically significant differences observed between the pre- and post-stimulation windows, where no ongoing active or sham stimulation was present. This, coupled with the findings of previous studies using online MRS acquired during stimulation (23, 24) suggest that interfence caused by ongoing intrascanner tDCS during spectral acquisition is not a likely source of error.

Another potential explanation for the null findings of this experiment is insufficient power as a result of too few participants. An analysis conducted in G*Power (42) determined

there were enough participants to detect at least a medium sized effect (i.e., effect size > 0.6, 1-$\beta = 0.8$, $\alpha = 0.05$). Many of the studies that have previously investigated biochemical effects of tDCS have noted significant findings with smaller sample sizes than the 19 used in this study, including $N = 12$ (22), $N = 17$ (23), and $N = 11$ (21). To this end it is believed that the study was sufficiently powered, in terms of the participant sample size, to detect a comparable effect. One of the problems with statistical power as outlined by Button et al. (43) is that while problems of low statistical power are typically associated with reduced chances of detecting a true effect, they may also reduce the likelihood of a statistically significant result being indicative of a true effect. That is, finding false positive effects due to inflated effect sizes. As Westwood et al. (40) illustrate, while it may be of value to include more participants in future studies, it calls the effectiveness of a single session of tDCS into question if the effects are so small. Referring to a meta-analysis in preparation, Westwood et al. (40) discuss an analysis of pooled studies looking at anodal stimulation in the frontal and temporal lobes which produced a sample size of almost 200 participants in which there was still no evidence of an effect of a single session of tDCS. In light of this, it is not believed that an increased sample size would have improved the outcome of this experiment.

One problem affecting the spectroscopy aspect of this study is that of how to quantify metabolite levels. Typical methods make use of water as an endogenous reference, or report the concentration as a ratio relative to an internal reference such as creatine or NAA. While creatine is typically favored as an internal reference (44) its use is complicated when using the MEGA-PRESS sequence as creatine signals are eliminated during subtraction and are not present in the difference edited spectrum, though they may be recovered from the spectra acquired without an editing pulse (commonly referred to as the "OFF" spectrum in the spectral pairs used to create the difference spectrum). NAA was not used as an internal reference as it has been demonstrated to be affected by anodal tDCS (22, 23), although no changes in NAA levels were measured over the course of the acquisition. The use of water as an endogenous reference can be problematic for studies such as this that attempt to measure metabolic changes in a dynamic manner, i.e., in relation to activity over time, as MRS signals have been shown to be susceptible to line-broadening artifacts associated with local BOLD effects (36). Using a fixed water reference taken at the end of the acquisition, as was done in this study, the reference signal was not subject to fluctuations as the result of a BOLD effect throughout the scan as the metabolites of interest were, i.e., comparing an unchanging reference to a signal subject to interference may increase the likelihood of a false change being detected. As a single, fixed water reference was used, it is difficult to decisively rule out any incidental BOLD-related fluctuations. However, such fluctuations would likely be manifest across all metabolites in the FWHM estimate given by LCModel, which is not seen in our data (**Tables 2**, **3**), making it unlikely to be a significant source of error. Ideally, an experiment such as this would benefit from the use of external referencing, such as the Electronic Reference To access *in vivo* Concentrations (ERETIC) method (45, 46).

In interpreting these findings, it is important to consider that tDCS is regarded as a neuromodulatory technique, it does not induce activity or action potentials, but rather facilitates increases or decreases in neuronal excitability. Bikson and Rahman (47) discuss the idea of activity-selectivity and task-specific modulation, that is, that tDCS will preferentially modulate a neuronal network that is already active, while not modulating a separate network that is inactive. One of the problems with the region of interest in this study is that it contains the primary auditory cortex and adjacent areas responsible for the sensation of sound and processing of speech (48). While other paradigms have investigated cortical areas that may be associated with a task, e.g., the primary motor cortex and force adaptation task (20), that may distinguish between blocks of activity and rest, the auditory cortex will experience ongoing sensory input during scanning. It is possible that no biochemical changes were observed between blocks as the local cortical circuit was already in an active state during the pre-stimulation window and that tDCS was not able to drive a higher level of activity.

In conclusion, using continuous online MRS, no significant change in the levels of Glx, GABA, or NAA in the left pSTG was observed that could be attributed to an effect of active, anodal tDCS. Despite this, the method provides a useful insight into the acute effects of stimulation paradigms and their effect on local neuronal circuitry. Further research investigating an effect of tDCS in this area suggests performing a similar experiment using cathodal tDCS, redesigning the experiment to allow 20 min of stimulation, perhaps combining this experiment with computer models and also using an external referencing method to avoid possible confounding variables associated with how metabolite levels are measured.

AUTHOR CONTRIBUTIONS

GD, AC, MH, KK, KH, and RG were involved in the conception and design of the study. GD, AC, MH, and KK contributed to planning and performing of the experiments. AC performed the spectral analysis component of this study. JA performed statistical analyses. LE contributed to acquisition of MR-Spectra. GD wrote the manuscript with the assistance and critical feedback of all contributing authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2018.01145/full#supplementary-material

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Conflict of Interest Statement: We wish to draw to the attention of the Editor that co-authors AC, LE, KH, and RG have shares in the company NordicNeuroLab A/S which produces add-on equipment for MRI examinations that were used in this study.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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