Research in this thesis was supported by a VIDI grant from the Netherlands Organization for Scientific Research and a Utrecht University High Potential Grant to prof. dr. H.E. Hulshoff Pol.

ISBN 978-94-6108-521-4

Lay-out and cover: Jobbe Holtes

Printed by: Gildeprint Drukkerijen, Enschede, The Netherlands

GLUTAMATE AND GABA IN SCHIZOPHRENIA

Glutamaat en GABA bij schizofrenie

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op donderdag 24 oktober 2013 des ochtends te 10.30 uur

door

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geboren op 17 mei 1982
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Introduction

Schizophrenia

Schizophrenia is a severe chronic psychiatric disease affecting approximately 1% of the population. The disease is characterized by abnormal mental functions and disturbed behavior, appearing in late adolescence or early adulthood. There are three classes of clinical features observed in schizophrenia, i.e. positive symptoms, including delusions, hallucinations and thought disorganization; negative symptoms, referring to deficits in emotional response and loss of motivation; and disturbances in cognitive functioning [1]. Structural findings point out that enlargement of the brain ventricles occurs, together with a progressive loss of brain tissue, mostly in the prefrontal cortex, hippocampus and thalamus [2-9]. This tissue loss does not seem to be caused by a loss of neurons but rather a reduced size of dendrites and axons -the structures that respectively receive and transmit information from electric and chemical signals from the neuronal body- and a reduced number of synapses -the connections between neurons where information is being transferred- [10].

Schizophrenia was first described in the late nineteenth century by Kraepelin as dementia praecox or premature dementia [11]. In the early twentieth century, Bleuler introduced the term schizophrenia, from the Greek words σ χ ι ζ ε ι ν -schizein, to split- and φ ρ η ν -phren, brain or mind-, emphasizing that he illness is not a dementia, i.e. leading to mental deterioration, but a fundamental disorder of dissociation of thoughts and feelings [12;13]. With the emergence of neuroleptic drugs in the second half of the twentieth century, the view of schizophrenia switched from a disorder caused by abnormal childhood to a neurochemically driven disease[13]. In fact, the finding that most effective antipsychotic drugs are dopamine receptor antagonists and that dopamine-releasing agents may induce psychosis led to the postulation of the dopamine hypothesis of schizophrenia [10;13]. Schizophrenia symptoms might thus be caused by dopamine overactivity, either because of increased dopamine levels or increased sensitivity to dopamine due to higher densities of dopamine receptors [10;14]. However, evidence on the nature of dopamine overactivity and the causal role of dopamine in schizophrenia remain inconclusive. Dopamine receptor densities are indeed elevated in schizophrenia, but it is not clear to which degree antipsychotic medication contributes to this [10]. Also, antipsychotics do not have an effect on cognitive impairments and negative symptoms, while the severity of these disease characteristics is correlated with the degree of cortical atrophy and ventricular enlargement [15;16].

The glutamate hypothesis

A focus on the cognitive symptoms of schizophrenia led to the more recent glutamate hypothesis or NMDA-receptor hypofunction hypothesis [13]. Glutamate is the most important excitatory neurotransmitter in the central nervous system and is synthesized in glutamatergic axon terminals from α-ketoglutarate -an intermediate of the citric acid cycle- or glutamine. Glutamatergic neurons connect brain regions -the prefrontal cortex, hippocampus and thalamus- that exhibit structural and functional abnormalities in schizophrenia [7] and the NMDA-type of glutamate receptor has an important role in brain functions that are impaired in schizophrenia such as the regulation of neuronal migration, neuronal differentiation, response to growth factors, development of dendrites and functional plasticity [15]. A possible mechanism behind the morphological changes and cognitive and behavioral disturbances observed in schizophrenia might be diminished activation of the NMDA receptors, since NMDA receptor antagonists produce similar symptoms as those seen in schizophrenia [17-19]. Administration of low doses of ketamine, an NMDA receptor antagonist, into healthy subjects produce negative symptoms that are specifically associated with schizophrenia; positive symptoms as a reaction on ketamine administration occur only in antipsychotic-free schizophrenia patients [15;20;21]. Low doses of ketamine also produce abnormalities in information processing in healthy subjects that are commonly seen in schizophrenia [15;22;23].

The glutamate hypothesis and dopamine hypothesis are not mutually exclusive as NMDA receptor hypofunction can lead to dopamine overactivity: NMDA receptor hypofunction leads to insufficient excitatory activity, disrupting the function of GABAergic interneurons, which may result in downregulated inhibitory activity and ultimately dopamine overactivity [24;25]. GABA (γ-aminobutyric acid) is the major inhibitory neurotransmitter in the central nervous system and is synthesized by decarboxylation of glutamate. GABAergic interneurons inhibit pyramidal neurons. Indeed, accumulating evidence suggests that abnormalities in pre- and postsynaptic components of GABAergic inhibitory neurotransmission are present in schizophrenia [21;26;27]. One of the most consistent findings is a reduction of mRNA levels encoding for the 67 kDA isoform of glutamic acid decarboxylase (GAD67), the enzyme that catalyzes decarboxylation of glutamate to synthesize GABA. Furthermore, mRNA levels encoding for the transporter protein that is responsible for the reuptake of GABA in the presynaptic neuron -GABA-transporter-1 (GAT-1)- are decreased [28;29], and expression of subunits of the GABA receptor that produces most of the physiological actions of GABA -the $GABA_A$ receptor- is altered [30].

In vivo measurement of NMDA receptor function is challenging, however, glutamate and GABA can be measured in vivo in the brain using magnetic resonance spectroscopy (MRS).

Magnetic resonance spectroscopy

Magnetic resonance spectroscopy (MRS) is a non-invasive technique that can be used to study the molecular composition of tissues *in vivo* and to identify metabolites involved in physiological or pathological processes. MR is based on the quantum mechanical concept of nuclear spin. Magnetic nuclei having an odd number of protons or neutrons; ^{1}H, ^{13}C, ^{19}F, ^{31}P and ^{23}Na are commonly used in MR experiments. The ^{1}H (proton) nucleus is the most commonly studied nucleus in clinical MRS studies of the brain. ^{1}H-MR spectra include (amongst others) signal information of N-acetylaspartate (NAA), creatine (Cr), choline (Cho), lactate (Lac), *myo*-inositol (mI) and glutamatergic compounds (glutamate (Glu), glutamine (Gln) and γ -aminobutyric acid (GABA)) [31;32].

When placed in a strong magnetic field (B_0), e.g. a MR scanner, the nuclear magnetization aligns itself along the direction of the applied field. When an additional magnetic field (B_1) -a radiofrequency (RF) pulse- is applied, the nucleus absorbs energy and its axis temporarily changes orientation. When the RF pulse is switched of, the nucleus tends to realign its axis to the direction of the major magnetic field, thereby emitting energy and inducing an observable signal (**Figure 1**).

Nuclei of the same element precess at a specific frequency, i.e. the Larmor frequency. This resonance frequency is highly sensitive to the experienced local magnetic field. Because of the variations in local magnetic fields generated by electrons surrounding the nucleus, the same nucleus resonates at slightly different frequencies in different molecules. Hence, the actual resonance frequency depends on the chemical environment of the nucleus. This phenomenon is referred to as chemical shift and gives rise to a spectrum of different resonance peaks, thereby allowing identification of different molecules using radiofrequency (RF) pulses. In MR spectroscopy it is custom to express this difference in frequency in parts per million (ppm) - a relative measure which is independent of the B_0 field strength. One nucleus can give rise to a resonance signal that is split into several smaller lines, which is caused by adjacent nuclei within the molecule affecting the magnetic field experienced by the nucleus. This is called J-coupling [31;32] and plays an important role in for example GABA measurements. Resonance signals can be processed using a Fourier transformation to yield a magnetic resonance spectrum (**Figure 2**).

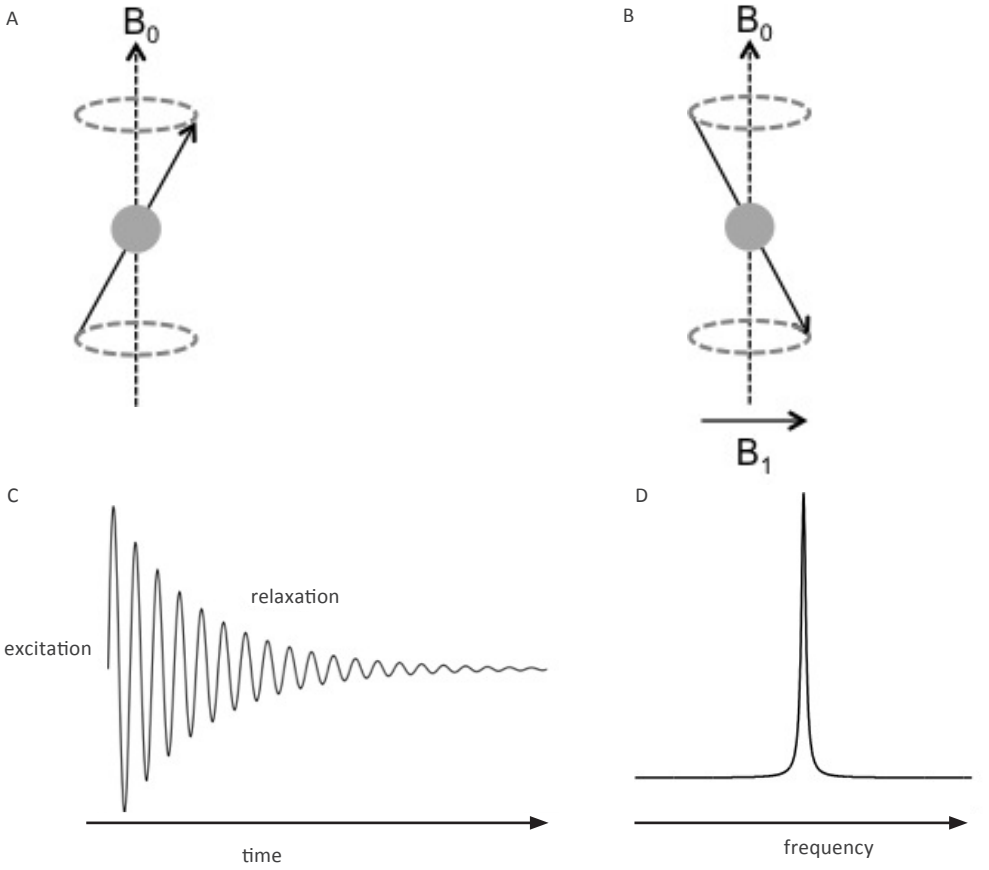

Figure 1: (A) When placed in a strong magnetic field, the nucleic axis of rotation precesses around the applied field (B_0). (B) A radio frequency (RF) pulse (applied in the direction perpendicular to the main magnetic field and denoted by B_1) changes the orientation of the nucleic axis. The angle of the rotation depends on the duration of the RF pulse. After the RF pulse, the nucleus returns to its ground state and precesses back around B_0, inducing a current, which can be detected. (C) As the nucleus returns to its start position, the induced current decreases, which is called the free induction decay (FID). (D) The FID can be Fourier transformed from the time domain to the frequency domain.

Since hydrogen-containing neurochemicals are present in much lower concentrations then water, the water signal covers up the signals from metabolites. To remove the water signal, which obscures the signal from the other metabolites, water suppression methods are used to obtain a ^{1}H-MR spectrum containing only neurochemicals [31;32].

To perform an MRS study, a strong homogeneous magnet and an RF coil for excitation and detection of the signal are needed. A subject lies in the center of the bore superconducting magnet with a birdcage antenna placed over the part of the body that is examined. An anatomical MR image is first acquired in order to accurately localize the volume of interest, or voxel, for MRS. The methods that

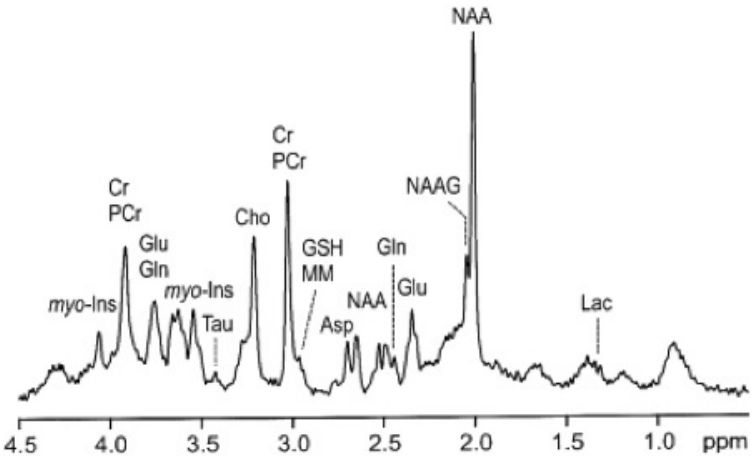

Figure 2: In vivo ^{1}H-MR spectrum of the human brain measured at a magnetic field strength of 7T (adapted from Tkáč et al. [57]).

are most commonly used for MRS are Point RESolved Spectroscopy (PRESS) and STimulated Echo Acquisition Mode (STEAM). PRESS is suited for the detection of metabolites that relax slowly after excitation with an RF pulse, and thus have long relaxation times (T_2). STEAM is better suited for detection of metabolites with short T_2 [31;32]. Since glutamate has a short T_2, STEAM would be the method of choice. However, STEAM suffers from significant loss of signal-to-noise ratio (SNR) because only half of the available signal can be obtained. With semi-Localization by Adiabatic SElective Refocusing (sLASER) the full glutamate signal can be acquired at a relatively short echo time (TE) [33].

^{1}H-MRS of glutamate

Glutamate is present in the brain in concentrations of 6-12.5 mM, with differences between gray and white matter [34;35]. Glutamate has two methylene groups and a methine group that are strongly coupled, resulting in a complex spectrum with low intensities of individual resonance peaks in the 2.0-2.4 ppm range and at 3.7 ppm in the ^{1}H-MR spectrum (**Figure 3**) [35].

In vivo glutamate measurement is complicated at lower magnetic field strengths. Due to its overlap with glutamine the individual signal contributions of both metabolites collapse into a single resonance, leading to the observation of the combined glutamate and glutamine (Glx) signals [35]. At magnetic field strengths of 7T and higher, the glutamate and glutamine resonances in the 2.0-2.4 ppm range become separate [34], which makes it possible to examine glutamate individually (**Figure 4**).

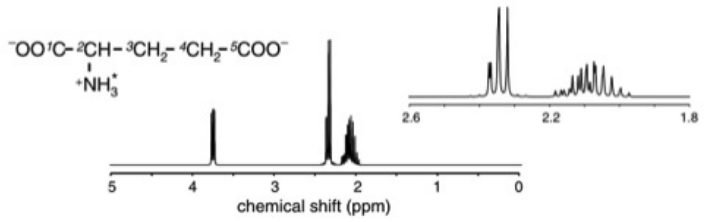

Figure 3: Chemical structure and 1H-MR spectrum of glutamate. Glutamate has five observable protons in two methylene (CH2) groups and one methine (CH) group. The proton of the 2CH group gives rise to a so-called doublet-of-doublets at 3.75 ppm. The four protons in the 3CH2 and 4CH2 groups give rise to multiplets between 2.04 and 2.35 ppm (adapted from De Graaf 34).

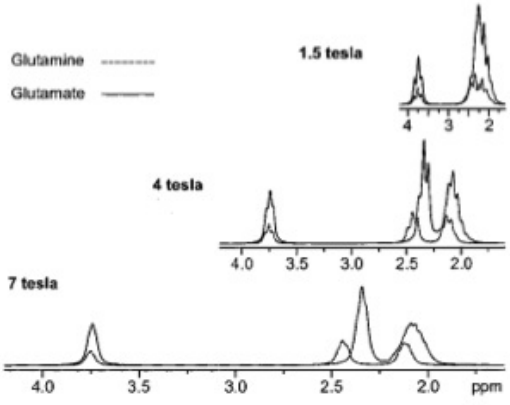

Figure 4: [1]H-MR spectra of glutamate and glutamine at different magnetic field strengths. At 7T, the 2.35 ppm resonance from glutamate is separated from the glutamine resonance (adapted from Tkáč et al. [57]).

[1]H-MRS of GABA

GABA is present in the brain at a concentration of approx. 1 mM, and has resonances at 1.9, 2.3 and 3.0 ppm in the [1]H-MR spectrum (**Figure 5**).

Measurement of GABA is usually carried out using spectral editing techniques [34], since its resonance peaks are overlapped by more intense resonance signals from other more abundant metabolites. Using spectral editing it is possible to separate the GABA signal from other signals since the GABA resonance at 3.01 ppm is coupled to the resonance at 1.89 ppm whereas other signals around 3.0 ppm are not coupled to signals around 1.9 ppm. Here spectral editing utilizes an RF pulse that only affects signals around 1.9 ppm that will have an effect on the 3.01 ppm resonance of GABA but not on other signals around 3.0 ppm. When a spectrum that is obtained using this frequency-selective RF pulse -or editing pulse- is subtracted from a spectrum obtained without this pulse, the difference spectrum -or edited spectrum- only contains signals that are affected by this pulse. The edited spectrum

will thus contain signals around 1.9 ppm and signals remote from 1.9 ppm that arise due to coupling effects (**Figure 6**) [35].

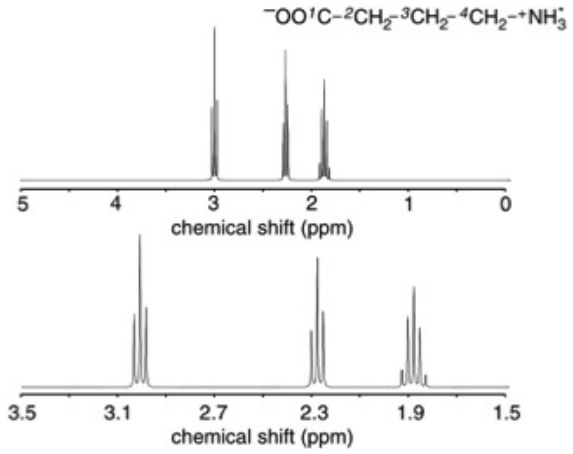

Figure 5: Chemical structure and ^{1}H-MR spectrum of GABA. GABA has six observable protons in three methylene (CH_2) groups. The protons of the $^{2}CH_2$ and $^{4}CH_2$ groups give rise to triplets at 2.28 ppm and 3.01 ppm. The proton of the 3CH2 group gives rise to the quintet at 1.89 ppm (adapted from De Graaf [34]).

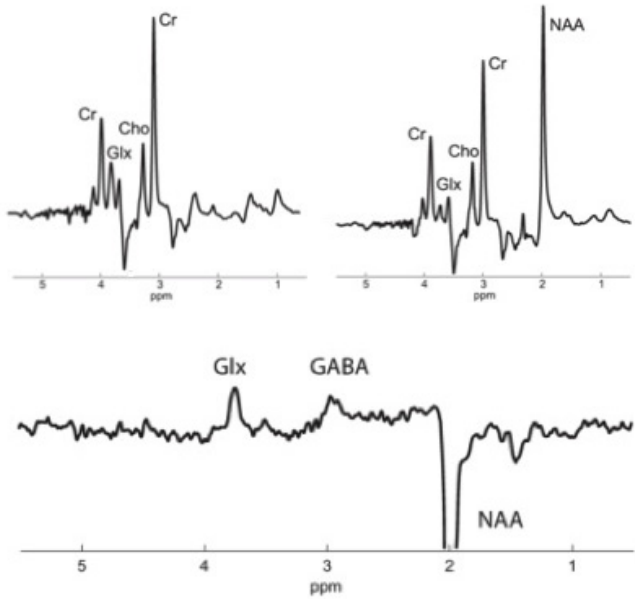

Figure 6: Editing of GABA in the human brain *in vivo* at a magnetic field strength of 7T. The top left spectrum is obtained with a RF pulse affecting signals close to 1.9 ppm. The top right spectrum is obtained without a frequency-selective RF pulse. By subtracting the first from the latter spectrum the edited spectrum (bottom) is obtained. The 3.01 ppm GABA resonance as well as the co-edited 3.75 ppm Glx resonance is visible. The inverted NAA signal at 2.0 ppm is present because it is suppressed by the frequency-selective RF pulse and is not subtracted away. The absence of a choline residue at 3.2 ppm indicates a good suppression of non-edited resonances (adapted from Andreychenko et al. [58]).

^{1}H-MRS of other brain metabolites

Although the focus in this thesis in on glutamate and GABA, the major spectral components NAA, creatine and choline may be worth mentioning as they could provide supporting evidence for the etiology of schizophrenia.

N-acetyl aspartate (NAA)

NAA is a free amino acid in the brain and present at concentrations of 7-16 mM, with higher concentrations in gray than in white matter. The most prominent resonance in the ^{1}H-MR spectrum is the NAA peak at 2.0 ppm, which arises from the methyl group in the acetyl moiety. The aspartate moiety gives rise to several smaller resonance peaks between 2.0 and 8.0 ppm [34;35]. NAA is a precursor for synthesis of *N*-acetyl aspartyl glutamate (NAAG), the most prevalent and widely abundant neuropeptide that may have a role in excitatory neurotransmission [36;37]. The NAA signal mostly represents the combined signals of NAA and NAAG, which may serve as a marker for neuronal health, viability and/or number of neurons and functional capacity of neuronal mitochondria [38].

NAA levels are reduced in several brain tissues in schizophrenia, with the most prominent reductions in the frontal lobe, hippocampus, temporal lobe and thalamus, and with little difference between first-episode and chronic schizophrenia [39;40]. However, in individuals at risk for schizophrenia, the most prominent reductions were found in the thalamus, and not in the frontal or temporal lobe [40]. Lower frontal NAA levels in first-episode patients are associated with an earlier age of disease onset, which supports the general notice that an earlier onset age might be related with a more severe illness progression and more marked anatomical changes [40-42]. Moreover, there seems to be an inverse correlation between NAA levels and negative symptoms [40;43;44].

Treatment with antipsychotic medication can alter NAA levels, with atypical antipsychotics resulting in less reduced, possibly increased, NAA levels as compared to typical antipsychotics [39;40;45-50].

Creatine (Cr)

Creatine and phosphocreatine (PCr) play an important role in energy metabolism and are present in the brain in concentrations of 4.5-6.0 mM for creatine and 4.0-5.5 mM for phosphocreatine, with higher concentrations in gray matter as compared to white matter.

The methyl and methylene protons of creatine and phosphocreatine give rise to resonance peaks in the ^{1}H-MR spectrum at 3.0 ppm and 3.9 ppm respectively.

Creatine is phosphorylated by creatine kinase (CK) in presence of adenosine triphosphate (ATP), which can in turn be regenerated from phosphocreatine in presence of adenosine diphosphate (ADP). Unlike ATP and ADP, creatine and phosphocreatine can diffuse rapidly across subcellular regions, providing transport of energy from regions of energy production to regions of energy consumption [38;51]. Total creatine concentrations in the brain are relatively stable, hence it is frequently used as an internal concentration reference [34;35]. However, in disease the creatine signal may alter and creatine referencing could become disadvantageous.

Alterations in creatine levels in schizophrenia have been reported, but observations are inconsistent and sometimes contradictory. Moreover, numerous studies have not detected changes in creatine levels in schizophrenia at all [52]. With regard to phosphocreatine, low frontal levels were observed in patients with high hostility-suspiciousness scores and high anxiety-depression scores [53], and high frontal levels were observed in patients with high negative symptom scores [54]. Asymmetry of phosphocreatine and phosphocreatine/ATP in the temporal lobes is also associated with symptom severity [55;56].

Choline (Cho)

Choline-containing compounds give rise to a prominent resonance at 3.2 ppm in the ^{1}H-MR spectrum. These choline-containing compounds, i.e. glycerophosphorylcholine (GPC), phosphorylcholine (PC) and free choline, are involved in phospholipid synthesis and degradation. As such, the choline signal reflects membrane turnover and alterations in the signal are associated with changes in membrane composition. Choline is also required for the synthesis of the neurotransmitter acetylcholine (ACh). Total choline concentrations in the brain are 1-2 mM, but since the contribution of the various choline-containing compounds to the choline peak is uncertain, it is difficult to interpret changes in the signal. Choline-containing compounds are also present in myelin and cell membranes in the brain in high concentrations, however, these compounds are not freely mobile and thus do not directly contribute to the choline signal [34;35;38]. Thus far, no consistent patterns of abnormalities in choline have been found in patients with schizophrenia [38].

Aim of this thesis

Proton magnetic resonance spectroscopy at a field strength of 7 tesla gives the opportunity to measure pure glutamate instead of Glx, the sum of glutamate and glutamine, which is measured at lower field strengths. In contrast to the dopamine hypothesis, with the glutamate hypothesis it is possible to explain, at least in part, the cognitive deficits that are observed in schizophrenia. Glutamate as well as GABA levels are likely to be reduced in schizophrenia. With an ultra-high field strength of 7T, it is now possible to accurately measure glutamate and GABA. The aim of this thesis is to investigate if glutamate and GABA levels are indeed reduced in patients with schizophrenia as compared to healthy subjects, using proton magnetic resonance spectroscopy at a field strength of 7T.

Outline of this thesis

Chapter 2 provides a review and meta-analysis of ^{1}H-MRS studies of glutamate, glutamine and Glx in schizophrenia until 2011. A systematic search identified twenty-eight studies including a total of 647 patients and 608 healthy controls. Meta-analyses show that frontal glutamate levels are decreased and frontal glutamine is increased in patients with schizophrenia as compared to healthy subjects. Moreover, glutamate and glutamine levels decrease faster with age as compared to healthy subjects, possibly reflecting ongoing aberrant processes in schizophrenia. **Chapter 3** reviews findings of post-mortem studies, animal models and human studies investigating pre- and postsynaptic components of GABAergic neurotransmission in schizophrenia. Studies have consistently found a reduction of mRNA encoding for the 67 kD isotype of glutamic acid decarboxylase (GAD67), the enzyme that catalyses the synthesis of GABA from glutamate, particularly in parvalbumin-containing GABAergic neurons. However, the current literature on ^{1}H-MRS studies of GABA in schizophrenia is scarce and inconsistent.

As postulated in chapter 2, glutamate progressively declines with age in patients with schizophrenia. However, since the studies described in chapter 2 used

magnetic field strengths up to 4 tesla, the glutamate resonance may not have been reliably measured. Moreover, investigating glutamate levels in normal maturation may contribute to elucidating the role of glutamate in schizophrenia. In **Chapter 4**, age-associated glutamate changes were measured in the medial frontal cortex of healthy young adults between 18 and 31 years of age. Glutamate concentrations in the medial frontal cortex decline with age in this group of healthy subjects, which is in line with grey matter thinning in this brain region but cannot be explained by cortical thinning alone. Furthermore, there are no alterations in NAA -a marker of neuronal integrity- and creatine -a marker of energy metabolism-, suggesting thedecrease in glutamate maybe due to physiological changes rather than anatomical changes.

In **Chapter 5**, the reproducibility of glutamate measurement in the human brain by two ^{1}H-MRS sequences (STEAM and sLASER) is assessed. The more recently developed sLASER sequence for 7 tesla shows twice as much signal as can be obtained using STEAM and benefits from improved localization accuracy. Eight age-matched healthy subjects were scanned twice using both STEAM and sLASER in the frontal and occipital brain region. Indeed, sLASER shows higher intraclass correlations for glutamate concentration as compared to STEAM in both the frontal and occipital brain region.

Chapter 6 describes the first 7T ^{1}H-MRS study of glutamate and GABA in schizophrenia. 17 patients with schizophrenia and 23 matched healthy subjects were included in this study, measuring brain metabolite levels in the prefrontal and occipital cortices. Lower GABA levels in the prefrontal cortex are found in patients as compared to healthy subjects, which is strongly associated with the level of cognitive functioning. This suggests a role for GABA through altered inhibitory neurotransmission in the prefrontal cortex in schizophrenia. Moreover, this role for GABA may be specific, since no differences in levels of glutamate, NAA, creatine and choline were found in these patients.

Chapter 7 presents a 7T ^{1}H-MRS study of glutamate and GABA associated with level of intelligence. The hypothesis that minimizing energy resources is beneficial to intelligence in the prefrontal and occipital cortices by measuring glutamate and GABA levels is tested in 23 healthy subjects. Higher glutamate levels and lower GABA levels in the occipital cortex are associated with higher working memory index, suggesting less and more efficient energy use in this part of the brain is beneficial for working memory as one of the markers for intelligence.

Finally, in **Chapter 8** the results of chapters 2, 3, 4, 5, 6 and 7 are discussed.

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2

Glutamate in schizophrenia: a focused review and meta-analysis of ^{1}H-MRS studies

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Schizophrenia Bulletin 2013; 39(1): 120-129

Abstract

Schizophrenia is a severe chronic psychiatric illness, characterized by hallucinations and delusions. Decreased brain volumes have been observed in the disease, although the origin of these changes is unknown. Changes in the NMDA-receptor mediated glutamatergic neurotransmission are implicated, since it is hypothesized that NMDA-receptor dysfunction in schizophrenia leads to increased glutamate release, which can have excitotoxic effects. However, the magnitude and extent of changes in glutamatergic metabolites in schizophrenia are not clear. With ^{1}H magnetic resonance spectroscopy (^{1}H-MRS), *in vivo* information about glutamate and glutamine concentrations can be obtained in the brain. A systematic search through the MEDLINE database was conducted to identify relevant ^{1}H-MRS studies that examined differences in glutamate and glutamine concentrations between patients with schizophrenia and healthy control subjects. Twenty-eight studies were identified and included a total of 647 patients with schizophrenia and 608 healthy control subjects. For each study, Cohen's *d* was calculated and main effects for group analyses were performed using the random-effects model. Medial frontal region glutamate was decreased and glutamine was increased in patients with schizophrenia as compared to healthy individuals. Group-by-age associations revealed that in patients with schizophrenia, glutamate and glutamine concentrations decreased at a faster rate with age as compared to healthy controls. This could reflect aberrant processes in schizophrenia, such as altered synaptic activity, changed glutamate receptor functioning, abnormal glutamine-glutamate cycling, or dysfunctional glutamate transport.

Introduction

Schizophrenia is a severe chronic psychiatric disease, characterized by hallucinations and delusions, starting in late adolescence or early adulthood. Structural MRI studies have established that schizophrenia is a brain disease with approximately a 3% tissue loss [1]. Moreover, this loss of brain tissue appears to be progressive as suggested by numerous longitudinal MRI studies [2-7]. It may be explained by reduced neuropil rather than by neuronal loss, implying changes in synaptic, dendritic and axonal organization in schizophrenia. Reduced neuropil has been observed mostly in the prefrontal region, hippocampus and thalamus [8]. This observation, together with the progressive brain tissue loss found in patients suggests that synaptic plasticity and cortical microcircuitry may be abnormal in schizophrenia. Interestingly, progressive brain changes are also present in discordant co-twins of patients with schizophrenia, suggesting that these changes are familial, and possibly genetic, and can therefore not solely be explained by antipsychotic medication intake [9].

The progressive loss of brain tissue in schizophrenia may represent an ongoing pathophysiological process, which could be an important target for therapeutic intervention. One of the possible mechanisms that may be involved is dysfunction of the glutamatergic system (**Figure 1**), which might affect synaptic plasticity and cortical microcircuitry, in particular NMDA-receptor signaling [10]. NMDA (*N*-methyl-D-aspartate) receptors are glutamate-gated ion channels, which play an important role in excitatory neurotransmission, plasticity and excitotoxicity [11;12]. Indeed, NMDA-receptor antagonists, such as ketamine and phencyclidine (PCP), produce symptoms that mimic psychosis as seen in schizophrenia [13-16].. Depending on the severity and duration of the NMDA-receptor hypofunction state, postsynaptic neurons can develop morphological changes and may cause chronic psychosis and structural brain changes [17-19]. However, *in vivo* measurement of NMDA-receptor function has been challenging. Although its agonist glutamate can be measured *in vivo*, evidence of aberrant glutamate levels in schizophrenia is inconsistent. It has been suggested that glutamate levels decrease with age in healthy individuals [20], but it is not known whether glutamate levels change with longer illness duration. Glutamate can be measured using ^{1}H magnetic resonance spectroscopy (^{1}H-MRS). ^{1}H-MRS allows *in vivo* assessment of the chemical composition of tissues in a non-invasive manner, by using the magnetic resonance signal of hydrogen to determine the concentrations of metabolites. With ^{1}H-MRS, both physiologically active and inactive glutamate are measured. The majority of physiologically active glutamate is derived from glutamine, therefore high levels of glutamine may suggest high

glutamatergic activity. The glutamatergic transmission and metabolism are strongly coupled, and findings on glutamate and glutamine levels in schizophrenia could give an insight in possible changes in neuronal activity during the course of the disease[21]. The aims of this structured review and meta-analysis were therefore to determine the extent to which glutamatergic changes occur in patients with schizophrenia compared to those of healthy controls and whether, if present, these changes become more pronounced with increasing age. For this purpose, we evaluated ^{1}H-MRS studies investigating glutamatergic metabolites in patients with schizophrenia.

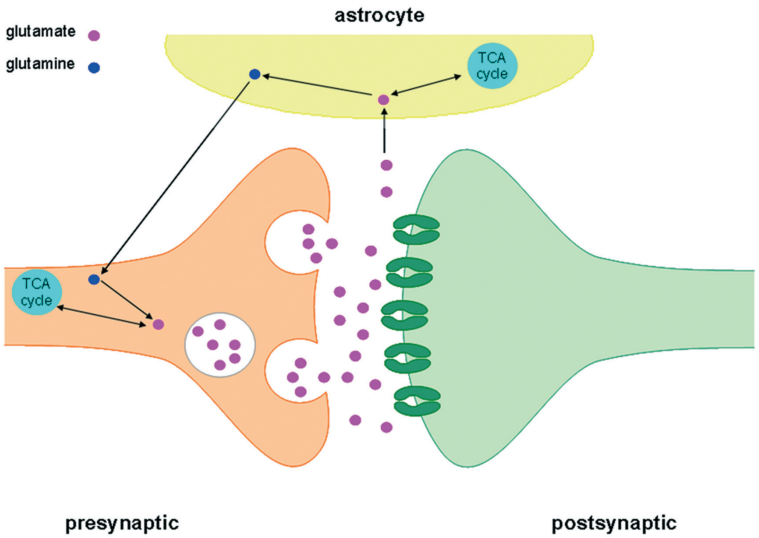

Figure 1: The glutamatergic synapse. Glutamate is an amino acid, a building block for proteins, therefore it is abundant in all cells of the body. It is also the most important excitatory neurotransmitter in the central nervous system (CNS). Glutamate is synthesized in axon terminals of glutamatergic neurons. It can be produced from α-ketoglutarate -a tricarboxylic acid (TCA)-cycle intermediate- or from glutamine. For glutamate synthesis from glutamine, the enzyme glutamine synthase is transported to the axon terminal. In the cytosol, it converts glutamine into glutamate. Transporters then concentrate glutamate in vesicles. Release of glutamate is triggered by influx of calcium (Ca2+) into the presynaptic neuron. The synaptic vesicles fuse with the cell membrane and release glutamate into the synaptic cleft. Glutamate is taken up by the postsynaptic neuron, by glia, or it is recycled in the presynaptic neuron.

Methods

Data sources

^{1}H magnetic resonance spectroscopy (^{1}H-MRS) studies that examined differences in glutamate levels between patients with schizophrenia and healthy control subjects were obtained through a MEDLINE search, using the keywords "glutamate", "spectroscopy" and "schizophrenia". Titles and abstracts of the articles were examined to see whether they fulfilled the inclusion criteria.

Study selection

Studies were included if they (1) used ^{1}H-MRS to examine glutamate concentrations in schizophrenia, (2) compared patients with a healthy control group, (3) did not use any interventions (i.e. were not subjected to certain actions or imposed to certain behaviors at the time of the study; patients taking antipsychotic medication were included), and (4) were published in the English language. Twenty-eight articles, published between 1994 and 2011, met these criteria. The magnetic field of the MRI-scanners used varied between 1.5 and 4.0 Tesla. The numbers of patients varied between 9 and 40 and the number of healthy controls varied between 11 and 46. Studies were divided based on the stage of illness of their patient populations into high risk, first-episode and chronic patient studies.

Three studies reported on individuals with a high genetic risk of schizophrenia [22-24]. One study reported on individuals with prodromal symptoms of psychosis [25]. Twelve studies reported on first-episode (FE) patients [26-37]. Eight of these studies report on a patient group of which the majority was antipsychotic-naïve [26;27;30-35]. In three studies on antipsychotic-naïve first-episode patients, (part of) the patient group was rescanned after receiving antipsychotic treatment [32;34;35]. In four studies on first-episode patients, the majority of patients had received antipsychotic medication [28;29;36;37]. Four studies report on first-episode patients and chronically ill patients [30-32;37].

Thirteen studies reported on chronically ill patients [30-32;37-44], of which one study only included antipsychotic-naïve patients [39], one study also included unaffected co-twins of patients [40] and one study also assessed gender differences [42]. One study reported on a sample consisting of both first-episode and chronically ill patients [45].

For two studies, the stage of illness of the patient population could not be determined [46;47] (**Table 1**). These two studies did provide all the information that was necessary for the meta-analysis, and were thus included.

Tabel 1: Study overview

Source	Field strength	Controls	Patients	Area	Disease stage	Medication
Bartha et al.[26]	1.5T	10	14	Frontal	FE	none
Bartha et al. [27]	1.5T	11	11	Temporal	FE	none
Bustillo et al. [28]	4.0T	10	14	Frontal	FE	atypical
Bustillo et al. [37]	4.0T	28	30	Whole brain (1 slice)	FE + Chronic	?
Chang et al. [38]	4.0T	22	23	Frontal, temporal, occipital	Chronic	atypical
Choe et al. [39]	1.5T	22	23	Frontal	Chronic	none
Galińska et al. [29]	1.5T	19	30	Frontal, temporal, thalamus	FE	atypical
Keshavan et al. [22]	1.5T	46	40	Frontal, temporal, parietal, occipital, basal ganglia	High risk	none
Lutkenhoff et al. [40]	3.0T	21	9 12	Frontal, hippocampus	Chronic Co-twins	? none
Ohrmann et al. [30]	1.5T	20	18 21	Frontal	FE Chronic	none atypical
Ohrmann et al. [31]	1.5T	20	15 20	Frontal	FE Chronic	none atypical
Olbrich et al. [36]	2.0T	32	9	Frontal, hippocampus, amygdala	FE	atypical
Öngür et al. [41]	4.0T	21	17	Frontal, occipital	Chronic	atypical
Reid et al. [48]	3.0T	23	26	Frontal	Chronic	atypical
Rowland et al. [49]	3.0T	11	20	Frontal, parietal	Chronic	atypical
Rüsch et al. [45]	2.0T	31	29	Frontal, hippocampus	FE + Chronic	atypical
Shirayama et al. [46]	3.0T	18	19	Frontal	?	atypical
Stanley et al. [32]	1.5T	24	11 10 11	Frontal	FE drug-naive FE medicated Chronic	none ? ?
Stone et al. [25]	3.0T	27	27	Frontal, hippocampus, thalamus	Prodromal	none
Tayoshi et al. [42]	3.0T	25	30	Frontal, basal ganglia	Chronic	?
Tebartz van Elst et al. [43]	2.0T	32	21	Frontal, hippocampus	Chronic	atypical
Théberge et al. [33]	4.0T	21	21	Frontal, thalamus	FE	none
Théberge et al. [44]	4.0T	21	21	Frontal, thalamus	Chronic	atypical/ typical
Théberge et al. [34]	4.0T	16	16 16 16	Frontal, thalamus	FE drug-naive FE 10M medicated FE 30M medicated	none atypical atypical
Tibbo et al. [23]	3.0T	22	20	Frontal	High risk	none
Wood et al. [47]	3.0T	14	15	Frontal	?	atypical
Wood et al. [35]	3.0T	19	15 19	Temporal	FE drug-naive FE medicated	none atypical
Yoo et al. [24]	1.5T	22	22	Frontal, thalamus	High risk	none

Two studies reported insufficient information to calculate Cohen's *d*, and these studies were excluded from the meta-analysis [22;32].

In total, 19 studies report on medial (pre)frontal brain areas (including the anterior cingulate cortex)[22;24-28;28;29;33;34;38-42;44;46-49], 7 studies report on the dorsolateral prefrontal cortex[24;30-32;36;43;45], five studies report on temporal brain areas[22;27;29;35;38], five studies report on the hippocampus[25;36;40;43;45], eight studies report on the thalamus[22;24;25;28;29;33;34;44], three studies report on occipital brain areas[22;38;41], two studies report on basal ganglia[22;42], two studies report on parietal brain areas[22;49], and one study reports results from a whole-brain slice[37]. Glutamatergic metabolites that entered the meta-analysis were glutamate, the major excitatory neurotransmitter in the central nervous system (CNS), glutamine, a glutamate precursor and glx, the sum of glutamate and glutamine. Meta-analysis was performed if three or more studies on glutamate, glutamine or glx were available in a particular brain region. Thus, a meta-analysis was performed on glutamate, glutamine and glx levels in the medial (pre)frontal region, and only on glutamate levels in the thalamus and hippocampus. Two studies reported on the same group of subjects [33;34], and to prevent potential bias of overlap we decided to include only one study [33] in the meta-analysis.

Data extraction

Within a meta-analysis one defines an effect size statistic, representing the quantitative findings of a set of research studies in a standardized form that permits meaningful comparison and analyses across the studies [50]. For each study in this meta-analysis, the effects size statistic Cohen's *d* was calculated [51]. The Cohen's *d* is the difference between the mean of the experimental group and the mean of the control group divided by the pooled standard deviation. The Cohen's *d* was calculated as follows. The mean concentration of glutamate, glutamine or glx for patients were subtracted from mean concentration for comparison subjects and divided by the pooled standard deviation of both. When means and standard deviations were not available, *d*-values were calculated from exact p-values, t-values or F-values. After computing individual effect sizes for each study, meta-analytic methods were applied to obtain a combined effect size, which indicated the magnitude of the association across all studies [52]. Meta-analyses and meta-regressions were performed with a random-effects model using the statistical package Comprehensive Meta-Analysis V2[53]. A random-effects model assumes that the effect size estimated by different studies varies among studies not only

because of coincidence but also because of differences in samples or paradigms and that these effect sizes have a normal distribution[54]. A *t*-test was subsequently performed on the null hypothesis that the *d*-value is 0.00, i.e. indicating no significant difference between the patient and control populations.. According to Cohen, absolute *d*-values of 0.2 represent small effects, values between 0.4 and 0.6 moderate effects, and *d*-values of 0.8 or higher large effects [51].In addition, heterogeneity was tested using Cochran's *Q* and I^2 [55;56]. When significant heterogeneity was found, meta-regressions of experimental variables were performed to investigate potential sources of heterogeneity and to identify possible outliers.

When data were available, we also performed a meta-analysis and meta-regression on N-acetyl aspartate (NAA), a potential marker of neuronal viability, to see if changes in glutamate, glutamine or glx could be a consequence of a decrease in functioning of neurons. A meta-analysis and meta-regression, when data were available, were performed on the gln/glu ratio to explore the changes of glutamate and glutamine relative to each other.

Results

Meta-analysis of the medial frontal region

Glutamate

The meta-analysis on glutamate included 9 studies with a total of 166 patients and 171 healthy controls [25;26;28;33;40-42;44-46]. The glutamate level in the frontal region was lower in patients than in controls (combined effect Cohen's *d* = -0.391, p=0.006) (**Figure 2A**).

Glutamine

The meta-analysis on glutamine included 8 studies with a total of 140 patients and 135 healthy controls [25;26;28;33;41;42;44-46]. The glutamine level in the frontal region was higher in patients than in controls (combined-effect Cohen's *d* = 0.403, p=0.045) (**Figure 3A**).

Glx (glutamate + glutamine)

The meta-analysis on glx included 8 studies with a total of 186 patients and 144 healthy controls [24;25;29;38;39;47-49]. The glx level in the frontal region was lower in patients than in controls but this finding was not significant (combined-effect Cohen's *d* = 0.122, p=0.393) (**Figure 4**).

Gln/glu ratio

The meta-analysis on the gln/glu ratio included 6 studies with a total of 112 patients and 116 healthy controls [28;33;41;42;44;46]. The gln/glu ratio in the frontal region was higher in patients than in controls but this was not significant (combined-effect Cohen's *d* = 0.308, p=0.062) (**Figure 5A**)

NAA

The meta-analysis on NAA based on the studies included in this meta-analysis, included 19 studies with a total of 401 patients and 378 healthy controls[24-26;28-31;33;38-42;44-49]. NAA in the frontal region was lower in patients than in controls (combined-effect Cohen's *d* = -0.320, p=0.019) (**Figure 6A**). The meta-analysis on the glu/NAA ratio included 7 studies [26;28;33;40-42;44]. The glu/NAA ratio in the frontal region was lower in patients than in controls (combined-effect Cohen's *d* = -0.357, p=0.038).

Meta-regression of age on effect size in the medial frontal region

Glutamate

The meta-regression of age on effect size across 9 studies with a total of 166 patients and 171 healthy controls showed a progressive decrease with age of glutamate in the frontal region in patients as compared to healthy controls [25;26;28;33;41;42;44-46] (p=0.008) (**Figure 2B**).

Glutamine

The meta-regression of age on effect size across 8 studies with a total of 140 patients and 135 healthy controls showed a progressive decrease with age of glutamine in the frontal region in patients as compared to healthy controls [25;26;28;33;41;42;44-46] (p=0.0005) (**Figure 3B**).

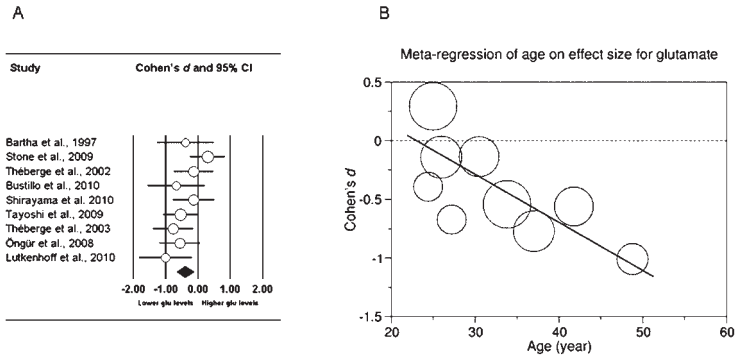

Figure 2: (A) Meta-analysis on frontal region glutamate. Displayed are the Cohen's *d* and 95% confidence interval per study. The size of the circles is proportional to the study's weight in the meta-analysis. Studies are ordered according to mean age of the patient group, from young to old. Data were recorded from the following anatomical regions: anterior cingulate cortex (ACC) (Stone et al. [25], Théberge et al. [33,44], Bustillo et al. [28], Tayoshi et al. [42], Öngür et al. [41]), medial prefrontal cortex (MPFC) (Bartha et al. [26], Shirayama et al. [46], Lutkenhoff et al. [40]). The combined Cohen's *d* is -0.391 (p=0.006). (B) Meta-regression of age of patients on effect size in frontal region glutamate. Each circle represents one study; the size of the circles is proportional to the study's weight in the meta-regression. (Slope, -0.04; standard error of the slope, 0.015; p=0.008).

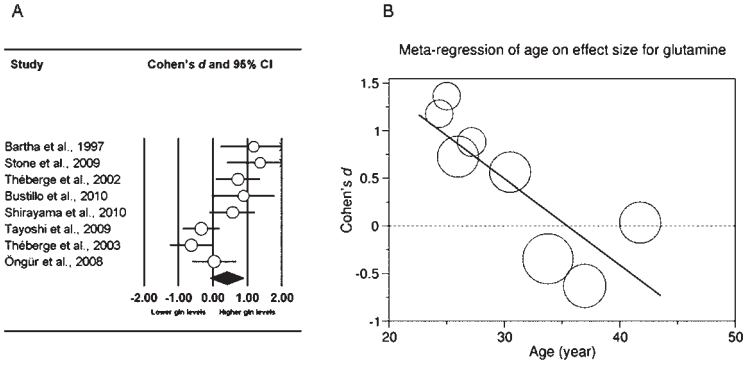

Figure 3: (A) Meta-analysis of frontal region glutamine. Displayed are the Cohen's *d* and 95% confidence interval per study. The size of the circles is proportional to the study's weight in the meta-analysis. Studies are ordered according to mean age of the patient group, from young to old. Data were recorded from the following anatomical regions: anterior cingulate cortex (ACC) (Stone et al. [25]; Théberge et al. [33,44]; Bustillo et al. [28]; Tayoshi et al. [42], Öngür et al. [41]), medial prefrontal cortex (MPFC) (Bartha et al. [26]; Shirayama et al. [46]). The combined Cohen's *d* is 0.403 (p=0.045). (B) Meta-regression of age of patients on effect size in frontal region glutamine. Each circle represents one study; the size of the circles is proportional to the study's weight in the meta-regression. (Slope, -0.1; standard error of the slope, 0.02; p=0.0005).

Gln/glu ratio

The meta-regression of age on effect size across 6 studies with a total of 112 patients and 116 healthy controls showed a progressive decrease with age of the gln/glu ratio in the frontal region in patients as compared to controls [28;33;41;42;44;46] (p=0.02) (**Figure 5B**).

NAA

The meta-regression of age on effect size across 19 studies with a total of 401 patients and 378 healthy controls showed a progressive decrease with age of NAA in the frontal region in patients as compared to controls [24-26;28-31;33;38-42;44-49] (p=0.04) (**Figure 6B**). The meta-regression of age on effect size across 7 studies with a total of 121 patients and 126 healthy controls showed a progressive decrease with age of the glu/NAA ratio in the frontal region in patients as compared to controls [26;28;33;40-42;44] (p=0.049).

Meta-analysis of the hippocampus

Glutamate

The meta-analysis of glutamate in the hippocampus included 3 studies, with a total of 47 patients and 60 healthy controls [25;40;45]. No differences in glutamate between patients and controls were observed (combined Cohen's d = 0.031, p=0.92) (**Figure 7**). There were not enough data available to perform a meta-analysis of glutamine in the hippocampus.

Meta-analysis of the thalamus

Glutamate

The meta-analysis of glutamate in the thalamus included 3 studies, with a total of 64 patients and 64 controls [25;33;44]. No significant difference was observed for glutamate between patients and controls (combined Cohen's d = -0.286, p=0.20) (**Figure 8**). There were not enough data available to perform a meta-analysis of glutamine in the thalamus.

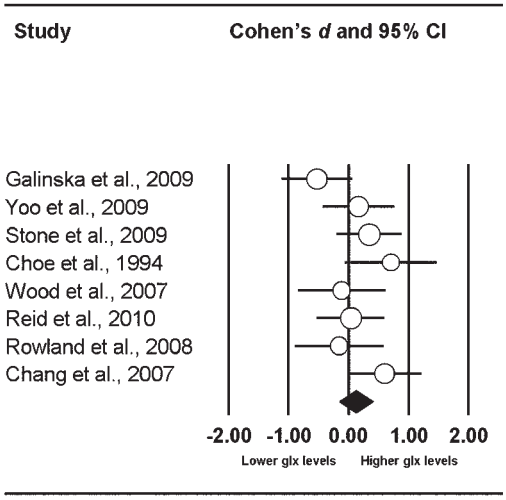

Figure 4: Meta-analysis on frontal region glx. Studies are ordered according to mean age of the patient group, from young to old. Data were recorded from the following anatomical regions: anterior cingulated cortex (ACC) (Stone et al. [25]; Wood et al. [47], Reid et al. [48]), dorsolateral prefrontal cortex (DLPFC) (Ohrmann et al. [30,31]), ACC + DLPFC (Yoo et al. [24]), medial prefrontal cortex (MPFC) (Rowland et al. [49]), frontal lobe (Galinska et al. [29]; Chang et al. [38]), prefrontal white matter (PFWM) (Choe et al. [39]). The combined Cohen's d is 0.122 (p=0.393).

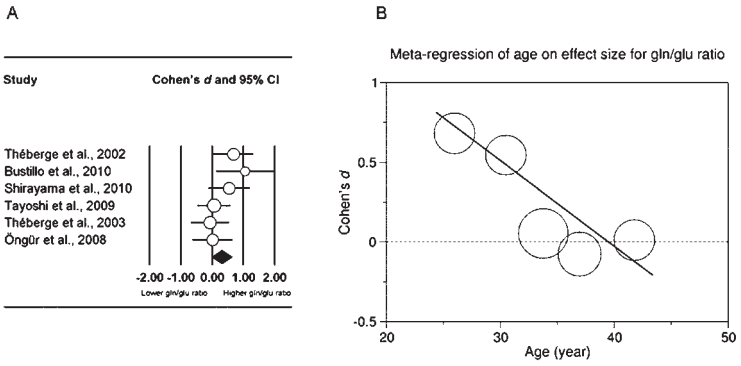

Figure 5: (A) Meta-analysis on frontal region gln/glu ratio. Displayed are the Cohen's d and 95% confidence interval per study. The size of the circles is proportional to the study's weight in the meta-analysis. Studies are ordered according to mean age of the patient group, from young to old. Data were recorded from the following anatomical regions: anterior cingulate cortex (ACC) (Théberge et al. [33,44], Bustillo et al. [28], Tayoshi et al. [42], Öngür et al. [41]) and medial prefrontal cortex (MPFC) (Shirayama et al. [46]). The combined Cohen's d is 0.308 (p=0.062). (B) Meta-regression of age on frontal region gln/glu ratio. Each circle represents one study; the size of the circles is proportional to the study's weight in the meta-regression. (Slope, -0.06; standard error of the slope, 0.025; p=0.02).

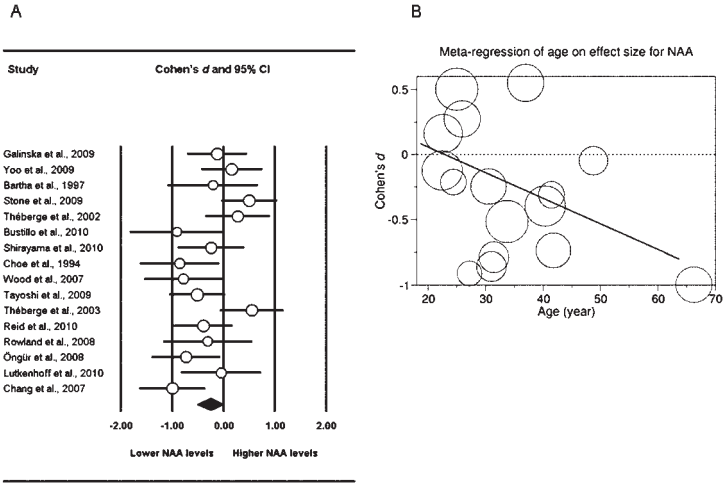

Figure 6: (A) Meta-analysis on frontal region NAA. Displayed are the Cohen's *d* and 95% confidence interval per study. The size of the circles is proportional to the study's weight in the meta-analysis. Studies are ordered according to mean age of the patient group, from young to old. Data were recorded from the following anatomical regions: anterior cingulate cortex (ACC) (Bustillo et al. [28]; Öngür et al. [41]; Reid et al. [48]; Stone et al. [25]; Tayoshi et al. [42]; Théberge et al. [33,44]; Yoo et al. [24]; Wood et al. [47]), medial prefrontal cortex (Bartha et al. [26]; Lutkenhoff et al. [40]; Rowland et al. [49]; Shirayama et al. [46]), frontal lobe (Galinska et al. [29]; Chang et al. [38]), prefrontal white matter (PFWM) (Choe et al. [39]) The combined Cohen's d is -0.260 (p=0.041). (B) Meta-regression of age on effect size in frontal region NAA. Each circle represents one study; the size of the circles is proportional to the study's weight in the meta-regression. (Slope, -0.02; standard error of the slope, 0.01; p=0.04).

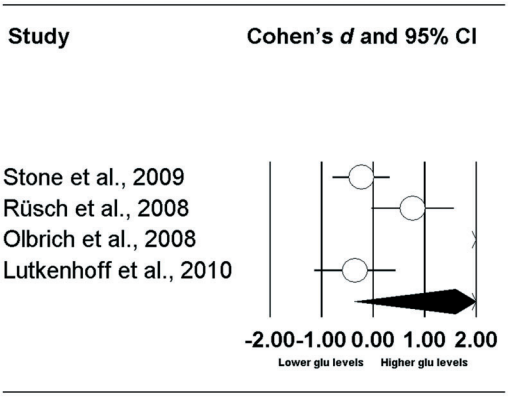

Figure 7: Meta-analysis on hippocampal glutamate. Displayed are the Cohen's *d* and 95% confidence interval per study. The size of the circles is proportional to the study's weight in the meta-analysis. Studies are ordered according to mean age of the patient group, from young to old. The combined Cohen's d is 1.605 (p=0.110).

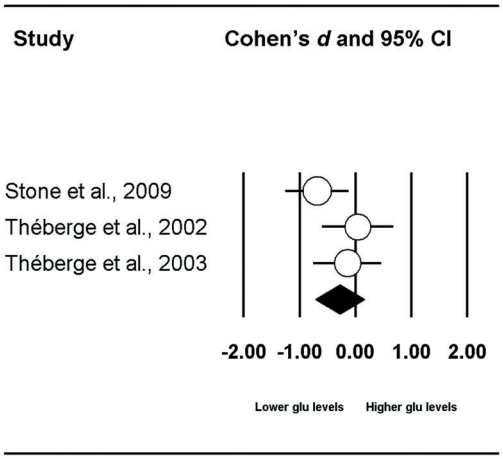

Figure 8: Meta-analysis on thalamic glutamate. Displayed are the Cohen's *d* and 95% confidence interval per study. The size of the circles is proportional to the study's weight in the meta-analysis. Studies are ordered according to mean age of the patient group, from young to old. The combined Cohen's *d* is -0.286 (p=0.197).

Outlier and sensitivity analysis

We performed an outlier and sensitivity analysis and found three potential confounding variables. First, lower magnetic field strengths account for more variation in Cohen's *d*. Second, the spectroscopic acquisition method STEAM (Stimulated Echo Acquisition Mode) shows less variation in Cohen's *d* then the acquisition method PRESS (Point-RESolved Spectroscopy) does. Third, shorter echo times account for more pronounced Cohen's *d* effects. For the meta-analyses on the medial frontal cortex we did not find any outliers.

Discussion

In this meta-analysis of ^{1}H magnetic resonance spectroscopy studies in schizophrenia, 24 studies were analyzed for changes in glutamate and glutamine. We found that frontal region glutamate is lower, and glutamine is higher in patients as compared to healthy controls. Interestingly, both glutamate and glutamine levels in the frontal region decrease progressively with age in patients with schizophrenia, which could suggest a progressive loss of synaptic activity. This is supported by the gln/glu ratio, which is increased in patients and declines with age. Also, we found decreasing NAA-levels in patients, which may be associated with the progressive brain volume reductions observed in patients with schizophrenia [2-7]. However, the altered frontal glutamate concentration in patients as compared to controls could not be solely explained by the changing NAA levels (with age). Thus, the findings from this meta-analysis suggests a decrease glutamate and increased glutamine contentration in the brain of patients with schizophrenia, particularly in older patients compared to older controls.

Several limitations have to be taken into account while interpreting the results of this meta-analysis. The most important limitation is that there is only a small number of studies that reported on glutamatergic changes in schizophrenia, as measured with *in vivo* ^{1}H magnetic resonance spectroscopy, of which most focused on frontal brain areas. It is of course possible that glutamatergic alterations only take place in the frontal region, and only in older patients, but the hippocampus and thalamus have been examined in only a few studies, as are the basal ganglia and temporal, parietal and occipital lobes. Differences in paradigms, such as magnetic field strength, acquisition mode, quantification method and frontal brain region, also have to be taken into account, since this could have an impact on the between-study variability [57]. Moreover, the progressive decline of glutamatergic levels observed has not been corrected for brain volume changes in this meta-analysis. Several, but not all, of the included studies did take into account partial voluming effects. Therefore we cannot completely exclude that some of the age-related decline in glutamate concentration in schizophrenia may have been due to brain volume changes. A longitudinal study which assessed patients when medication-naive and after treatment, found reductions in precuneal gray matter after 10 months and in frontal, temporal, parietal and limbic lobes after 30 months of treatment, which was correlated with thalamic glutamine reductions[34]. It is therefore too early to make definitive conclusions about the involvement of glutamate in schizophrenia.

Secondly, this meta-analysis only includes cross-sectional data. The conclusion that glutamatergic concentrations progressively decrease with age in schizophrenia is therefore preliminary. Also, the effect size statistic Cohen's *d* displays the deviation of patients as compared to healthy controls, which means that possible age-related changes in healthy controls are not taken into account in the meta-analysis. There is evidence though, that glutamate changes with age in the healthy brain [20]. Unfortunately, medication effects could not be taken into account in this analysis, because a major part of the studies did not report sufficient information on antipsychotic intake. The effects of antipsychotic medication cannot be discounted, since it has been suggested that haloperidol, clozapine and olanzapine cause reductions in glutamatergic levels in the rat brain [58]. However, long-lasting effects of antipsychotic medication have not been found in human studies so far[28;34].

Thirdly, in this meta-analysis we focused on glutamatergic neurotransmission, however, of course, the measurement of glutamatergic levels alone is not sufficient to draw conclusions about possible neurochemical alterations in patients with schizophrenia. Also, changes in glutamatergic levels may not be detectable with ^{1}H-MRS,. Glutamate and glutamine are difficult to distinguish at lower magnetic field strengths, which makes the quantification of glutamate and glutamine problematic[59]. Also, ^{1}H-MRS only provides information about the concentration of metabolites. Other metabolite levels, such as compounds involved in energy metabolism and cell proliferation, should also be taken into account. GABA is also likely to play an important role. An earlier meta-analysis made clear that N-acetylaspartate levels are reduced in patients with schizophrenia in frontal lobe gray and white matter [60].

In conclusion, glutamatergic levels appear to decrease progressively with age in patients with schizophrenia as compared to healthy controls. It is possible though, that these progressive reductions are, at least partly, caused by the decrease of brain volume or accumulative intake of antipsychotic medication. In addition, we find an overall increased glutamine level in patients with schizophrenia, also in the early (medication-naïve) phase of the disease. Indeed, from the meta-regression, it appears that glutamine levels drop below the healthy control level after patients have reached the age of 35 years, when the majority has reached the chronic phase of the illness. Thus, while we can only draw conclusions with great caution, increased glutamine levels could possibly represent an early marker for glutamate changes in schizophrenia. In fact, the gln/glu ratio is most prominently increased in patients with schizophrenia as compared to the controls at a young age and seems to normalize in older patients This may reflect a deficiency in glutaminase, the enzyme that converts glutamine into glutamate, which results in reduced

glutamate levels and increased glutamine levels in the frontal region [61]. In line with this, the amount of glutamate that is released into the synaptic cleft and taken up by astrocytes would decrease. In astrocytes, diminishing levels of glutamate are converted back into glutamine, which in the long term could result in reduced glutamine levels. However, we should be aware that while glutamine represents the major precursor for neuronal glutamate (and GABA), glutamate can also be synthesized from the tricarboxylic acid cycle [62] and changes in glutamate may also present changes in this second pathway.

There are several possible causes that may explain the altered glutamate levels in schizophrenia. In fact, diminished glutamate receptor activation, in particular of the NMDA-type of glutamate receptor, may be a possible underlying mechanism of reduced synaptic activity, since NMDA-receptor antagonists produce the same symptoms as those seen in schizophrenia [17;18;25]. The NMDA-receptor hypofunction hypothesis was evolved from studies with NMDA-receptor antagonists, such as phencyclidine (PCP) and ketamine[17]. Injection of these NMDA-receptor antagonists leads to increased glutamine levels and decreased glutamate levels[15;63;64] suggesting that NMDA-receptor blockade results in a shift in the glutamine-glutamate cycle[63]. Animal studies show that the absence of NMDA-receptor subunits can cause alterations at the molecular and behavioral level and produce schizophrenia-like symptoms [65-78]. This might suggest that changes in NMDA-receptor functioning play an important role in schizophrenia, supporting our current meta-results of age-related decreased glutamatergic levels in patients.

Diminished activation of NMDA-receptors results in insufficient excitatory activity, disrupting the monitoring function of GABAergic inhibitory neurons. In response, these inhibitory neurons may downregulate their own activity [79;80]. This results in disinhibition and hyperactivity of excitatory pathways, which will eventually cause neuronal damage[17]. GABA could thus be an important target for future human *in vivo* ^{1}H magnetic resonance spectroscopy research.

Knockout models suggest that other types of glutamate receptors may be involved in the pathophysiology of schizophrenia. There is evidence that knockout of AMPA (α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate) receptor subunits is implicated in psychosis-like behavior [81]. Deletion of subunits of metabotropic glutamate receptors induce glutamatergic function deficits [82;83] and schizophrenia-like symptoms [84;85].

Interestingly, mouse models have shown that a number of proteins associated with glutamate receptors, some of which directly linked with schizophrenia susceptibility genes, have been suggested to show modifications in patients, which in turn cause schizophrenia-like phenotypes [86;87]. Another cause of NMDA-

receptor hypofunction could be altered expression of intracellular and extracellular glutamate transporters [88-91]. Dysfunction of glutamate transport in the synaptic cleft, and also glutamate transport inside presynaptic neurons, can play a role in the altered glutamatergic levels. Changes in the membrane metabolism in schizophrenia may also be implicated in the changed concentrations of glutamate in schizophrenia. ^{31}Phosphorus magnetic resonance spectroscopy (^{31}P-MRS) has revealed alterations in membrane metabolism in schizophrenia. An increase in membrane breakdown products has been measured in first episode schizophrenia, while a reduction of membrane breakdown products has been measured in chronic schizophrenia[92-94]. Together with increased glutamatergic metabolites in early schizophrenia followed by decreased glutamatergic metabolites in chronic schizophrenia, this is consistent with an excitotoxic process[94].

More information is essential to make solid conclusions on glutamatergic changes over time in schizophrenia. Up until now, studies were mostly cross-sectional and investigated different brain areas. Also, studies did not control adequately for medication intake, duration of illness and severity of symptoms. It is thus important that future studies take into account the abovementioned limitations and are carried out in a more uniform manner in order to obtain accurate information about alterations in the glutamatergic system in schizophrenia.

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3

GABA neurotransmission in schizophrenia: what can ^{1}H-MRS contribute?

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In preparation

Abstract

Schizophrenia is a severe brain disease characterized by hallucinations, delusions, disorganizing thinking and impairments in cognitive functioning such as deficits in working memory. Evidence from post-mortem studies suggests that alterations in the cortical γ-aminobutyric acid (GABAergic) neurons contribute in part to the clinical features of schizophrenia. In vivo-measurement of GABA levels using magnetic resonance spectroscopy (MRS) offers the possibility to provide insights more directly in the relationship between deficit components of GABAergic neurotransmission, GABA-mediated inhibitory activity and clinical symptoms of the disease. This study reviews findings of post-mortem studies, animal models and human studies investigating pre- and postsynaptic components of GABAergic neurotransmission. Converging evidence implicates alterations in both presynaptic and postsynaptic components of GABA neurotransmission in schizophrenia. Thus, GABA may play an important role in the pathophysiology of schizophrenia, and MRS can provide more direct insights in the influence of GABA in early stages of the disease.

Introduction

Schizophrenia is a chronic psychiatric disease affecting approximately 1% of the world population [1]. The disease is characterized by hallucinations, delusions, disorganized thinking and by several cognitive deficits. Several lines of evidence suggest that abnormalities of specific cortical inhibitory neurons and its neurotransmitter γ-aminobutyric acid (GABA) could play an important role in the pathology of schizophrenia [2]. Indeed, GABA is responsible for the majority of synaptic inhibition in the central nervous system (CNS). Knowledge of GABA neurotransmission can thus be essential to the understanding of schizophrenia. However, current evidence about GABAergic abnormalities in schizophrenia has mostly been based on post-mortem studies and these studies have not yet provided a conclusive answer about GABAergic alterations and activity in schizophrenia. In-vivo measurements of GABA in schizophrenia may reveal additional insights. The aim of this article is to provide a brief introduction to GABA, review the findings of multiple post-mortem studies on different components of GABA neurotransmission and review the few in-vivo findings on GABA concentration in the brains of patients with schizophrenia that have been published so far using magnetic resonance spectroscopy (MRS).

GAD and GABA

GABA is formed from the decarboxylation of glutamate by glutamic acid decarboxylase (GAD) (Figure 1) [3]. Based on its molecular weight, it is possible to distinguish two isotypes of GAD, namely the 65 kD isotype GAD65 and the 67 kD isotype GAD67. Animal studies reveal that both isoforms are involved in different aspects of GABAergic neurotransmission [4]. Based on research in GAD knockout mice it was found that GAD67 seems to be responsible for the majority (80-90%) of GABA production; GAD67 knockout mice revealed decreased GABA content, while the GABA content remained the same in GAD65 knockout mice [5]. Moreover, GAD65 seems to be responsible for the rapid production of GABA during periods of high synaptic demand, whereas GAD67 is responsible for basal levels of GABA [6;7]

After synthesis in the presynaptic terminal of GABA neurons, GABA is packaged into vesicles by the vesicular GABA transporter (VGAT) which is embedded in the vesicular membrane [8]. A presynaptic action potential can subsequently induce a Ca^{2+}-mediated fusion of the vesicle membrane and the presynaptic membrane which leads to the release of GABA into the synaptic cleft. Alternatively, after strong

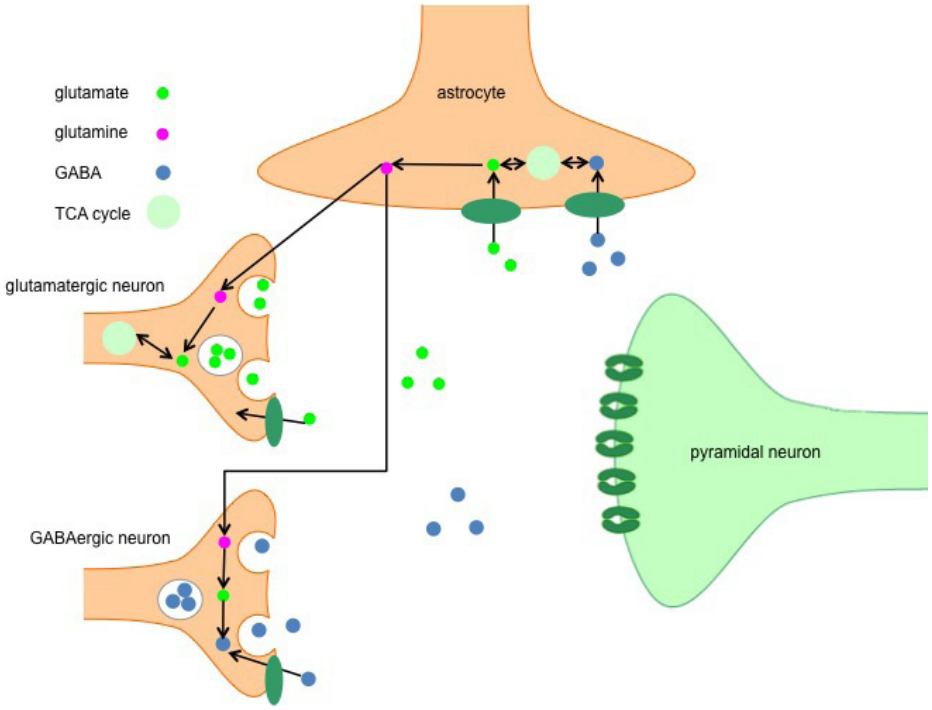

Figure 1: Metabolism of GABA. After synthesis in the presynaptic terminal of GABA neurons, GABA is packaged into vesicles by the vesicular GABA transporter (VGAT) which is embedded in the vesicular membrane 8. The synaptic activity of GABA is terminated when GABA is taken up by GABA transporters (GAT) embedded in the plasma membranes of neurons and astrocytes 9. When GABA is taken up by neurons it can be either repacked in vesicles for neurotransmission or it can be degraded by the enzyme GABA transaminase (GABA-T) to succinic semialdehyde (SSA). After conversion of SSA to succinate, it enters the TCA cycle and is subsequently converted into glutamate 9;16. The following conversion of glutamate to GABA by GAD65 and GAD67 completes the GABA cycle.

depolarization or altered ion homeostasis, specific GABA transporters (GAT) may reverse their direction resulting in non-vesicular release of GABA [8;9]. Since GAD65 is primarily located at axon terminals and synaptic vesicle membranes, it appears to be predominantly responsible for packaging and release of GABA. In contrast, GAD67, which resides in the cytosol (i.e. the liquid found inside cells), is associated with GABA synthesis and non-vesicular release [10;11].

After release into the synaptic cleft, GABA exerts its inhibitory activity by binding to two different types of receptors; $GABA_A$ and $GABA_B$ receptors. In contrast to the metabotropic $GABA_B$ receptors, $GABA_A$ receptors are ligand-gated chloride ion channels and produce most of the physiological actions of GABA [12]. $GABA_A$ receptors have a pentameric subunits structure derived from different gene families and include α, β, γ, δ, ε, π and θ subunits. Some of these subunits

have several isoforms (α 1-6, β 1-3 and γ 1-3) [13]. In most cases, the pentamers of subunits include a pair of α subunits and a pair of β subunits in combination with a fifth subunit (γ or δ) [14].

The synaptic activity of GABA is terminated when GABA is taken up by GABA transporters (GAT) embedded in the plasma membranes of neurons and astrocytes [9]. In humans, it is possible to distinguish four types of GABA transporters, namely GAT 1 to 3 and betaine GABA transporter (BGT-1). GAT-1 is characterized by a widespread expression within the brain and since it is primarily found on presynaptic GABA neurons, this transporter is predominantly responsible for reuptake of GABA into neurons [8;9]. The expression of GAT-3 is much more limited and since GAT-3 is located at astrocytes, it accounts primarily for reuptake into local astrocytes [15]. In contrast to GAT-1 and GAT-3, GAT-2 and BGT-1 play a very limited role in the direct GABAergic neurotransmission [9]. When GABA is taken up by neurons it can be either repacked in vesicles for neurotransmission or it can be degraded by the enzyme GABA transaminase (GABA-T) to succinic semialdehyde (SSA). After conversion of SSA to succinate, it enters the TCA cycle and is subsequently converted into glutamate [9;16]. The following conversion of glutamate to GABA by GAD65 and GAD67 completes the GABA cycle.

Of the GABA cycle, several aspects have been measured in schizophrenia, including post-mortem levels of GAT-1, GAD67, GAD65 and $GABA_A$ receptor subunits. In vivo MRS measurements have revealed concentrations of GABA in local brain areas non-invasively, that reflect several aspects of the GABA neurotransmission cycle. Thus, by studying post-mortem brain samples and MRS-imaging modalities, it is possible to describe alterations of the different components of the GABA cycle more precisely in patients with schizophrenia.

GAD67 and schizophrenia

One of the most consistent post-mortem findings in schizophrenia is a reduction of mRNA encoding for GAD67 in the dorsolateral prefrontal cortex (DLPFC) in layers 1 through 5, as based on twenty of the twenty-two published studies measuring GAD67 in the brains of patients (Table 1) [4;6;7;17-29]. The reduction of GAD67 mRNA seems to be accompanied by a reduction of GAD67 protein levels, although this has been less extensively studied (total of three studies) [7;18]. The mRNA and protein levels of GAD65 are not altered in the DLPFC in patients with schizophrenia [18;30]. Furthermore, the density of GAD65-positive axon terminals remains unchanged as well [31]. With only two studies demonstrating an increase of GAD67 mRNA [32;33], the reduction of GAD67 mRNA has been one of the most consistent findings

in postmortem studies on GABAergic neurotransmission in individuals with schizophrenia [34;35]. Thus, overall, there is evidence for a reduction of GAD67 in the brains of patients with schizophrenia.

Is the reduction in GAD67 in schizophrenia a state or a trait phenomenon? Post-mortem studies have associated GAD67 with information on severity and chronicity of the illness, cognitive impairment, or antipsychotic medication intake in patients. GAD67 levels did not differ between patients with or without characteristics predicting or reflecting a severe illness course in one study [7]. However, there is a variance of GAD67 mRNA levels across different schizophrenia patients and this variation might reflect different subtypes of schizophrenia [26;36]. In addition, in the two post-mortem studies finding an increase in GAD67 mRNA, it has been suggested that this was due to the more severe disease-related characteristics or older age of the study cohorts, as compared to the majority of post-mortem studies on GAD67 mRNA levels [32;33]. Importantly, expression of GAD67 is regulated in an activity-dependent manner and therefore, lower levels of GAD67 could be the consequence of reduced cortical activity due to psychiatric illness and/or cognitive impairment [37]. However, this seems unlikely, since GAD67 levels did not differ between patients with or without characteristics predicting or reflecting a severe illness course and thus the reduction of GAD67 in schizophrenia is more likely to reflect a trait phenomenon of the illness [7].

We have to take into consideration that the altered GAD67 mRNA in the prefrontal cortex (PFC) could be due to factors other than the pathological processes underlying schizophrenia, such as antipsychotic medication intake. Long-term exposure of antipsychotics in patients with schizophrenia may influence the expression of GAD67 mRNA. Studies in animals have pointed out that long-term treatment of dopamine D2-receptor antagonists, such as haloperidol, influence the expression of GAD67 mRNA in the basal ganglia [38-41]. However, the density of D2-receptors in the PFC is much lower than in the basal ganglia and animal studies demonstrate that long-term treatment with haloperidol or olanzapine did not affect the expression of GAD67 mRNA in the PFC [20;26]. Also, importantly, medication-naive patients revealed a similar reduction of GAD67 mRNA compared to patients that had received antipsychotic medication [26]. Thus, overall antipsychotic medication intake does not seem to explain the decrease in GAD67 in patients with schizophrenia.

The line of evidence discussed above indicates that post-mortem findings with regard to the gene expression of GAD67 appear not to be a consequence of the disease, but rather a component in the pathophysiology of schizophrenia.

Table 1: GAD67 in post-mortem brain in schizophrenia

Author	Brain region	Findings	Comments
Akbarian et al. [17]	DLPFC (BA9) - In situ hybridization	GAD67 mRNA ↓ (decreased subset)	- Overall neuronal density ~ - 30-50% decrease in layers I-VI of GAD67 mRNA positive neurons
Impagnatiello et al. [70]	STG (BA22)	GAD67 protein ↓ (decreased expression)	- 70% decrease GAD67 protein - RELN and RELN mRNA ↓ (≈50%) - GABAA receptor α1 en α5 subunit mRNA ↑
Benes et al. [31]	ACC (BA24) PFC (BA9)	GAD65-IR terminals ~	
Guidotti et al. [18]	DLPFC (BA9) - In situ hybridization - SZ + BD	GAD67 mRNA ↓ GAD67 protein ↓ (decreased expression)	- Overall neuronal density ~ - 68% decrease of GAD67 mRNA expression, 50% decrease of GAD67 protein expression - 50% decrease of RELN mRNA, 25-30% decrease of RELN positive cells in layer 1 - GAD65 protein ~
Mirnics et al. [19]	DLPFC (BA9) - cDNA microarrays	GAD67 mRNA 20-70% ↓ (decreased expression)	- 20-70% decreased expression
Volk et al. [20]	DLPFC (BA9) - In situ hybridization	GAD67 mRNA ↓ (decreased subset)	- 25-35% decrease in layers III-V of GAD67 mRNA positive neurons - Overall neuronal density ~
Hakak et al. [32]	DLFPC (BA46) - Affymetrix oligonucleotide microarrays	GAD 67 mRNA ↑ GAD65 mRNA ↑ (increased levels)	- Elderly patients
Knable et al. [21]	DLPFC (BA9)	GAD67 mRNA ↓	
Hashimoto et al. [6]	DLPFC (BA9) - In situ hybridization - cDNA microarray	GAD67 mRNA ↓ (decreased density)	- Decrease of PV mRNA expression in layer III and IV (no change in density of PV-IR neurons) - Decrease of GAD67 mRNA positive neurons - CR mRNA ~ - 50% of PV mRNA positive neurons lacks detectable levels of GAD67 mRNA
Dacheva et al. [33]	Prefrontal cortex (BA46) Primary visual cortex (BA17)	GAD67 mRNA ↑ GAD 65 mRNA ↑ (increased expression)	- Elderly patients - No concomitant GAD protein change
Woo et al. [22]	ACC (BA24) - BD+SZ	GAD67 mRNA ↓ (decreased denisity)	- 53% decrease in layer II and 28% decrease in layer V of GADG67 mRNA positive neurons
Hashimoto et al. [23]	DLFPC (BA9) - In situ hybridization	GAD67 mRNA ↓ (decreased expression)	- 24-48% decrease of GAD67 mRNA expression - Correlated BDNF /TrkB mRNA ↓
Fatemi et al. [100]	Cerebellar cortex - MD+SZ	GAD67 protein ↓ GAD65 protein ↓ (decreased expression)	Psychosis (SZ/BD)
Veldic et al. [24]	DLPFC (BA9) - BD + SZ	GAD67 mRNA ↓ (decreased density)	- Decrease in layers I and II of GAD67 mRNA positive neurons
Straub et al. [101]	DLPFC	GAD67 mRNA ↓ GAD67 protein ~	- GAD67 mRNA 19% ↓
Veldic et al. [29]	DLPFC (BA9)	GAD67 mRNA ↓ (density of GAD67 mRNA positive neurons)	Density reelin mRNA ↓
Woo et al. [25]	DLPFC (BA9)	GAD67 mRNA ↓ (density of GAD67 mRNA positive neurons)	- GAD67 mRNA ↓ in 27-36% of GABA cells in layers 2-5 - NR2A mRNA ↓ in GABA cells with unaltered GAD67 expression

Hashimoto et al. [26]	DLFPC (BA9)	GAD67 mRNA ↓	- SST subset - Transcript levels in order of magnitude of change: SST, GABAA α1 and δ, NPY, CCK, GAT-1, GAD67, GABAA γ2 + α4 ↓
Hashimoto et al. [27]	DLPFC (BA9) ACC (BA24) Primary motor cortex Primary visual cortex	GAD67 mRNA ↓	- GAT-1 mRNA, $GABA_A$ α1 and δ subunit, GAD65 mRNA, SST mRNA and PV mRNA ↓ - calretinin mRNA ~
Thompson et al. [4]	ACC (BA24) OFC (BA45), STG (BA22)	- GAD67 mRNA ↓ in OFC, ACC, STG, striatum (caudate nucleus and nucleus accumbens) and thalamus	- OFC: 30% decrease in layers III and IV - ACC: 15% in layer II, 23% in layer Va - STG: layer IV
Duncan et al. [28]	DLPFC (BA9/46)	GAD67 mRNA ↓	- 17% GAD67 mRNA ↓ - GABAA α5 mRNA ↓ - GABAA α1 + α2 mRNA ~
Curley et al. [7]	DLPFC	GAD67 mRNA ↓ GAD67 protein ↓	

Box 1: Subsets of GABAergic neurons

Based on molecular, morphological and physiological features, it is possible to distinguish various subsets of cortical GABA neurons, with the double bouquet, basket and chandelier cells being the most abundant cortical GABAergic interneurons subsets [2;75]. The subpopulations have different influences on the regulation of information processing in the DLPFC, partly because the axons of the GABAergic interneurons synapse at different locations on the pyramidal neuron [93;94]. Furthermore, it is possible to identify certain morphological and functional subgroups of GABA neurons which contain different calcium-binding proteins [48;95;96]. The chandelier GABA neurons synapse at the so-called axon initial segments (AIS) of pyramidal neurons and therefore provide inhibitory inputs to the AIS. These synaptic connections are formed in such a way that vertical arrays are formed, hence the name 'cartridges' [2;63]. Furthermore, these neurons contain the calcium-binding protein parvalbumin [6;97]. The axons of basket (or wide arbor) cells synapse at the cell bodies and proximal dendrites of pyramidal neurons. Similar to chandelier neurons, basket cells in the PFC contain the protein parvalbumin [48]. The double bouquet cells contain the calcium-binding proteins calbindin and target the distal dendrites of pyramidal neurons [2;91]. A third calcium-binding protein, calretinin, is expressed by approximately 50% GABAergic neurons in the DLPFC and this protein is mainly expressed by double bouquet cells [48] . Since the parvalbumin-containing chandelier and basket neurons synapse at the axon initial segment and soma respectively, they provide a much stronger inhibitory regulation of the pyramidal neurons than those neurons which synapse at distal dendrites, such as the double bouquet cells [98;99]. Thus given the heterogeneity in synaptic targets and specific features of the different subclasses, an impaired interaction between these subclasses and pyramidal neurons influence excitability and hence functional output in different manners.

Chandelier neurons (parvalbumin) → AIS (strong inhibition)
Basket/Wide-arbor neurons (parvalbumin) → soma (strong inhibition)
Double bouquet (calretinine/calbinidin) → distal dendrites (inhibition)

GAD67 and GABAergic neurons

It is unlikely that the reduction of GAD67 mRNA can be attributed to a decrease in the number of neurons in the PFC of patients with schizophrenia, because the majority of studies reported no change or an increase in neuron density [17;20;42;43]. Moreover, GAD67 mRNA is not decreased in all GABAergic neurons in the DLPFC in schizophrenia [6;17;20;22;24;25;29]. The expression of GAD67 mRNA was decreased below a detectable level in only a part of the neurons (25-35%), while the remaining neurons with an expression of GAD67 above the detectable level revealed mRNA levels similar to controls [20;25]. Thus, specifically, the density of neurons which expressed a detectable level of GAD67 mRNA was decreased [20]. Since the GAD67 mRNA expression is reduced in only a few layers of the DLPFC and since the density of neurons positive for GAD67 mRNA in the DLPFC is decreased without detectable neuronal loss, it is suggest that the impairments of GABAergic gene expression is limited to a certain subset of GABA-ergic neurons [20]. This subset could concern the chandelier, double bouquet or wide arbor neurons, which can be distinguished by the presence of specific calcium-binding proteins [2] (for a detailed explanation of subsets of GABAergic neurons, see **Box 1**).

This subset of GABA neurons appears to include the parvalbumin-containing GABAergic neurons in schizophrenia. The parvalbumin mRNA expression is reduced in layers III and IV, but not in layers II, V or VI, in the prefrontal cortex of patients with schizophrenia [6;20;25;35]. The overall expression of parvalbumin mRNA is decreased whereas the density of neurons expressing detectable levels of parvalbumin is unaltered [6;44;45]. This implies that the reduction of parvalbumin mRNA is not accompanied by a loss of parvalbumin containing neurons. The reduced parvalbumin mRNA expression is correlated with the decreased density of GAD67mRNA-positive GABAergic neurons. It seems that 50% of the parvalbumin-positive neurons lack detectable amounts of GAD67 mRNA [6], whereas calretinin mRNA expression and the density of calretinin positive neurons remain unchanged in schizophrenia [6;46]. These findings imply that the reduced GAD67 mRNA expression may be selective for the parvalbumin-containing subgroup of GABA neurons in the PFC [6]. Since the chandelier neurons and basket neurons are the only GABAergic neurons containing parvalbumin, these subclasses are likely to be involved in the pathophysiology of schizophrenia. Furthermore, the affected subpopulation may include the chandelier neurons, because the reduced densities of GAD67 mRNA are predominantly reduced in the same cortical layers as where the chandelier neurons are located [47].

The observed alterations regarding parvalbumin are not likely to be caused by exposure to antipsychotic medication. Long-term exposure to haloperidol and benztropine did not lead to an altered expression of parvalbumin mRNA [6]. Furthermore, transcript levels for parvalbumin were reduced to the same extent in the DLPFC of medication naïve individuals compared to patients receiving antipsychotic medication [26]. Finally, effects of alcohol abuse or use of benzodiazepines did not explain the findings [26;27].

However, other subclasses than the parvalbumin-containing neurons might contribute to the decreases of GAD67 mRNA in schizophrenia. GAD67 mRNA reductions are also seen in layer I, II and V of the DLPFC, and these layers are known to hardly contain parvalbumin-expressing GABA neurons [6;48]. Furthermore, the GAD67 mRNA reduction seen in layer I, II and V were not accompanied by any deficits in parvalbumin mRNA levels in one study [6]. Thus, while parvalbumin-expressing GABA neurons seem predominantly involved in the pathophysiology of schizophrenia other GABA neurons may also be implicated.

GAT-1 and schizophrenia

The transporter protein GAT-1 is present in the presynaptic neuron and is responsible for the synaptic reuptake of GABA [23;49]. Therefore, it terminates the synaptic activity of GABA and regulates the duration and efficacy of synaptic GABAergic neurotransmission [50]. The mRNA levels of the GABA-Transporter-1 (GAT1) are decreased in GABAergic neurons in the DLPFC (Table 2) [26;27;49;51]. In contrast to the reduced GAD67 and the consequent attenuation of inhibitory GABAergic neurotransmission, reduced GAT-1 levels suggest *increased* availability of GABA in the synapse [52]. Hence, it is unclear whether the net effect of the diminished synthesis and the declined re-uptake results in an increase or decrease of the inhibitory tone on pyramidal cells (for more information see Box 1) [52]. As for GAD67 mRNA, the diminished GAT-1 mRNA expression is reduced below detectable level in only a subset of GABAergic neurons and is relatively unaltered in the majority of the GABAergic neurons [49]. The affected subset appears to be the parvalbumin-containing neurons [2;49]. The reduction of GAT-1 mRNA expression is limited to layers II through V, the same layers in which parvalbumin containing neurons are found [49;53]. Furthermore, cartridges of the parvalbumin containing chandelier neurons can be identified by their immunoreactivity for GAT-1. Indeed, the density of GAT-1 immunoreactive cartridges is decreased, while markers of other axon terminal populations remain unchanged [53;54]. Since this lower density of GAT-1 immunoreactive cartridges reflecting decreased GAT-1 protein demonstrates

positive associations with the decreased GAT-1 mRNA levels, the reduced GAT-1 mRNA levels may account for the decreased density of GAT-1 immunoreactive axon cartridges in chandelier neurons [49]. Importantly, the reduction of GAT-1 immunoreactive cartridges is not attributable to a reduction of chandelier neurons, because the density of parvalbumin-immunoreactive neurons was unchanged [44;45]. Thus, the density of chandelier neurons containing GAT-1 protein in the DLPFC in patients with schizophrenia was significantly reduced whereas the density of parvalbumin containing neurons remains unaltered. This finding implies that the reduced levels of GAT-1 mRNA are limited to the chandelier neurons [25;53].

Similar is the reduction of GAD67 mRNA, the observed decline of GAT-1 mRNA expression is related to the disease process of schizophrenia and is not attributable to other factors associated with schizophrenia. First, the observations may not be attributable to long-term exposure to antipsychotic medication, because long-term exposure to therapeutic blood levels of haloperidol in monkeys did not result in changes in the expression of GAT-1 mRNA nor in the expression of GAT-1 protein [47;53;54]. Second, as mentioned above, since studies reported either an unchanged neuron density or an increased neuron density in the prefrontal cortex in patients with schizophrenia, the decrease of GAT-1 mRNA cannot be attributed to changes in neuron density [17;55]. Furthermore, mRNA of synaptophysin, a synaptic vesicle protein present in virtually every cortical neuron, did not reveal a change in expression levels indicating an unaltered amount of neurons [47;49]. Third, effects of alcohol abuse or use of benzodiazepines did not explain the findings [26;27].

Table 2: GAT-1 in post-mortem brain in schizophrenia

Author	Brain region	Findings	Comments
Woo et al. [53]	DLPFC (BA9)	GAT-1-IR cartridges of chandelier neurons ↓	- 40% decrease across layers 2-6 - axon terminals of other classes unaffected
Pierri et al. [54]	DLPFC (BA46)	GAT-1-IR cartridges of chandelier neurons ↓	
Ohnuma et al. [51]	DLPFC (BA9) (BA10)	GAT-1 mRNA ↓	$GABA_A$ receptor α1 subunit mRNA ↑
Volk et al. [49]	DLPFC (BA9)	GAT-1 mRNA ↓	21-33% decrease in layers 1-5 below detectable level in subset of GABA neurons
Konopaske et al. [50]	Auditory association cortex (BA42)	GAT-1-IR cartridges of chandelier neurons ~	
Hashimoto et al. [26]	DLPFC (BA9)	GAT-1 mRNA ↓	- Transcript levels in order of magnitude of change: SST, $GABA_A$ α1 and δ, NPY, CCK, GAT-1, GAD67, $GABA_A$ γ2 and α4 ↓
Hashimoto et al. [27]	DLPFC (BA9) ACC (BA24) Primary visual and motor cortices	GAT-1 mRNA ↓	- GAD67 mRNA, $GABA_A$ α1 and δ subunit, GAD65 mRNA, SST mRNA and PV mRNA ↓ - calretinin mRNA ~

Post-synaptic GABA receptors and schizophrenia

To determine whether there is an increase in GABAergic inhibition due to reduced re-uptake by means of GAT-1 or whether there is a decrease of GABAergic inhibition because of diminished synthesis by means of GAD67, it is required to assess potential alterations of post-synaptic GABA receptors. $GABA_A$ receptors are ligand-gated chloride ion channels and produce most of the physiological actions of GABA [12]. $GABA_A$ receptors have a pentameric subunit structure and the subunits are derived from different gene families encoding for different subunits including α, β, γ, δ, ε, π and θ (α 1-6, β 1-3 and γ 1-3) [13]. The pentamers of subunits include in most cases a pair of α subunits and a pair of β subunits in combination with a fifth subunit (γ or δ) [14]. Early studies demonstrated increased muscimol binding in pyramidal neuron cell bodies in patients with schizophrenia [56-58]. However, since muscimol recognizes all types of $GABA_A$ receptor subunits, advantages in recent technology has enabled the investigation of deficits of individual $GABA_A$ receptor subunits [59].

Specific α subunits are characterized by different subcellular localization within the central nervous system. Over 95% of the GABA-ergic synapses on the AIS of pyramidal neurons contain the α2 subunit, while only 15% of cortical GABA receptors contain the α2 subunit [60;61]. It appears that this subunit is characterized by a high affinity, a fast activation and a slow deactivation [62]. Since the above mentioned anatomic position and functional features, the $GABA_A$ α2-subunit serves as a major source for inhibitory tone on pyramidal neurons (for more information see Box 1) [63]. As mentioned before, parvalbumin-containing neurons, which seem to exhibit a reduced expression of GAT-1 and GAD67 mRNA, target the AIS of pyramidal neurons. Indeed, it has been demonstrated that the GABA α2 receptor subunit is up-regulated in the AIS of pyramidal neurons [57;63;64]. This compensatory increase in α2 subunit-density might take place in response to reduced extracellular GABA concentrations due to diminished GABA synthesis [2;63]. Furthermore, immunoreactive GAT-1 cartridges and the density of α2 subunits at the post-synaps of pyramidal neurons demonstrate an inverse correlation, which implies that $GABA_A$ α2 subunits are upregulated at the AIS of pyramidal neurons and GAT-1 is downregulated in order to provide a synergetic compensation for the diminished GABAergic activity [63]. Noteworthy, in contrast to GAD67 mRNA and GAT-1 mRNA, mRNA expression levels of post-synaptic $GABA_A$ α2-receptor subunits seem to be unaltered [17;28]. An explanation for the discrepancy between $GABA_A$ α2-receptor binding and mRNA expression of the GABA α2 subunit has not yet emerged [33]. However, as reported, the reduction of α2 receptor subunits are

exclusively found at the AIS synapses. The lack of upregulation of the mRNA of the $GABA_A$ receptor subunits might therefore be explained by the fact that inhibitory synapses at the AIS of the pyramidal neuron make up less than 10% of the total number of inhibitory synapses of the pyramidal neuron [17;65].

It seems that the mRNA levels of the $GABA_A$ $\alpha 1$, $\gamma 2$, $\alpha 4$, $\alpha 5$ and δ receptor subunits are downregulated in the DLPFC of patients with schizophrenia (**Table 3**) [17;26;27;66-69]. However, two studies reported an increase of $\alpha 1$ subunit mRNA expression [51;70], one study revealed an increase of $\alpha 5$ subunit mRNA [70], one study observed an increase of the $GABA_A$ receptor $\alpha 1$ subunit protein [59] and one study demonstrated no change of the $\alpha 4$ receptor subunit [68]. In contrast to the a2 subunit localized at the AIS of pyramidal neurons, $GABA_A$ receptors containing the $\alpha 1$, $\alpha 5$, $\gamma 2$ and δ (often co-expressed by $\alpha 4$) subunits are predominantly localized in the dendrites of pyramidal neurons [61;71;72].

Importantly, to assess the validation of these results it is important to exclude factors that could have influenced the findings. Several observations confirm that antipsychotic medication may not account for the observed alterations of post-synaptic $GABA_A$ receptors. The density of $\alpha 2$ subunits is increased regardless of medication use [63]. Furthermore, animal studies in which rats were exposed to benzodiazepines did not reveal changes in the expression level of $GABA_A$ receptor $\alpha 2$-subunit mRNA or protein and long-term exposure to haloperidol or olanzapine did not result in altered $\alpha 1$, $\alpha 2$, $\alpha 5$, $\beta 2$ or δ subunit mRNA levels [26;69;73]. Furthermore, the $\alpha 1$ and δ subunits were reduced to the same extent in the DLPFC of individuals who were not taking antipsychotic medication at the time of death [26]. The findings in $\alpha 1$ and δ subunits are not driven by the effects of alcohol abuse or by the use of benzodiazepines [26;27]. The observed alterations in the postsynaptic $GABA_A$-receptors do not seem to be a consequence of an increased number of neurons, because the majority of studies have reported no change or an increase in neuron density [20;35;42;43]. (For an overview of pre- and postsynaptic GABAergic alterations see (**Figure 2**).

Table 3: Post-synaptic receptor findings for GABA in post-mortem brain in schizophrenia

Author	Brain region	Findings	Comments
Hanada et al. [56]	DLPFC (BA9) and caudate nucleus	Musci-mol binding $GABA_A$ receptor pyramidal neuron bodies ↑	- GAD activity ~
Benes et al. [64]	ACC	Musci-mol binding GABAa receptor pyramidal neuron bodies ↑	- Increase in layer I-III of $GABA_A$ binding
Akbarian et al. [17]	DLPFC (BA9)	- $GABA_A$ α1-5 receptor mRNA subunits ~ - $GABA_A$ γ2 receptor subunit ~	- $GABA_A$ α5 and γ2 receptor subunit trend towards decrease
Benes et al. [57]	DLPFC (BA10)	Musci-mol binding GABAa receptor pyramidal neuron bodies ↑	- Increase in layer II, III, V and VI of receptor binding activity
Huntsman et al. [66]	DLPFC (BA9)	GABAa receptor γ2 subunit mRNA ↓	- $GABA_A$ γ2 receptor subunit trend towards decrease (28% decrease)
Impagnatiello et al. [70]	DLPFC (BA9)	- $GABA_A$ receptor α1 subunit mRNA ↑ - $GABA_A$ receptor α5 subunit mRNA ↑	- 70% decrease GAD67 protein - RELN and RELN mRNA ↓ (≈50%)
Dean et al. [58]	DLPFC (BA9)	Musci-mol binding $GABA_A$ receptor pyramidal neuron bodies ↑	
Ohnuma et al. [51]	DLPFC (BA9) BA10	$GABA_A$ receptor α1 subunit mRNA ↑	- Decrease in layer III and IV of α1 subunit - GAT-1 mRNA ↓
Mirnics et al. [19]	DLPFC (BA9)	$GABA_A$ receptor β1, γ2, π subunits	
Ishikawa et al. [59]	DLPFC (BA9)	- $GABA_A$ receptor subunit α1 and β2/3 ↑	
Ishikawa et al. [102]	DLPFC (BA9)	$GABA_B$ receptor 1 protein ↓	
Vawter et al. [67]	DLPFC (BA9 + BA46)	$GABA_A$ receptor δ subunit mRNA ↓	
Volk et al. [63]	PFC	Postsynaptic α2-subunit protein at AIS of pyramidal neuron↑	- 37% ↑ α2 protein expression - Inverse correlation with immunoreative cartridges for GAT-1
Hashimoto et al. [26]	DLPFC (BA9)	- Postsynaptic α1- and γ2 subunit mRNA ↓ Extra-synaptic δ- and α4 subunit mRNA ↓	- Transcript levels in order of magnitude of change: SST, $GABA_A$ α1 and δ, NPY, CCK, GAT-1, GAD67, $GABA_A$ γ2 and α4 ↓
Hashimoto et al. [27]	DLPFC (BA9) ACC (BA24) Primary visual and motor cortices	- Postsynaptic α1 and extrasynaptic δ subunit mRNA ↓	- GAD67 mRNA, GAT-1 mRNA, GAD65 mRNA, SST mRNA and PV mRNA ↓ - calretinin mRNA ~
Aviles et al. [68]	DLPFC (BA9)	- $GABA_A$ receptor δ mRNA expression ↓ - $GABA_A$ receptor α4 mRNA expression ~	- Possibly reflection of reduced $\alpha_1\beta_2$
Duncan et al. [28]	DLPFC (BA9/BA46)	- GABAa α5 mRNA ↓ - GABAa α1 + α2 mRNA ~	- GAD67 mRNA ↓ - From neonates to adults: 3x ↑ α1 mRNA, 1.5x ↓ α2 mRNA, 3x ↓ α5 mRNA
Beneyto et al. [69]	DLPFC	- $GABA_A$ α2 subunit mRNA ↑ - $GABA_A$ α1, α5, β2 subunit mRNA ↓ - $GABA_A$ α3, β1, β3 ~	- Increase in layer II of α2 subunit mRNA - Decrease in layer III and IV of α1 subunit mRNA - Possibly total number of α1β2γ2 receptor ↓

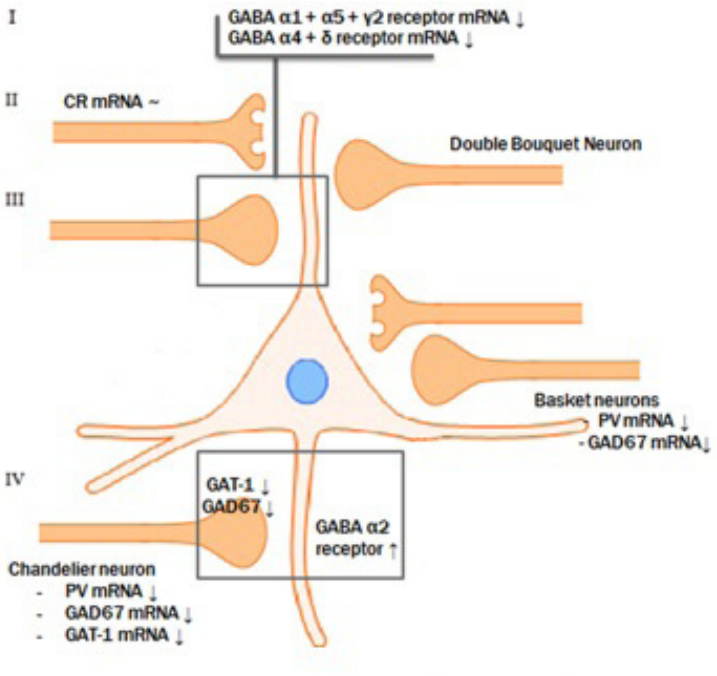

Figure 2: Pre- and postsynaptic GABAergic alterations. The reductions of GAD67 mRNA, PV mRNA and GAT-1 mRNA levels in the parvalbumin-containing chandelier neurons seem to result in a compensatory postsynpatic upregulation of α2-receptor at the axon initial segment of the pyramidal neuron. Presynaptic alterations in neurons targeting the dendritic domain of the pyramid neuron might also be accompanied by abnormalities of the postsynaptic GABA α1, α5 and γ2 and the extrasynaptic α4 and δ receptor subunits.

Pan-cortical

Schizophrenia is characterized by various symptoms, because individuals with schizophrenia suffer from cognitive impairments such as disturbances of working memory, and abnormalities in the field of affective, motor and sensory functioning. It seems there is sufficient histological-pathological evidence to link impairments in GABAergic neurotransmission in other cortical regions than the DLPFC to the pathology and cognitive dysfunction observed in schizophrenia [52].

Similar to the DLPFC, the anterior cingulate cortex (ACC), primary visual cortex and primary motor cortex were characterized by the same deficits in GABAergic gene expression as seen in the DLPFC. The largest declines were reported for the levels of mRNA encoding for parvalbumin [27]. These brain areas also exhibit a decrease of GAD67 mRNA, GAD65 mRNA, GAT-1 mRNA and $GABA_A$ receptor α 1 and δ subunits [4;22;27]. Calretinin levels remained unchanged [27]. Thus, the deficits in GABAergic gene expression in the DLPFC, with selective involvement

of parvalbumin containing subsets of GABA neurons, occur in a similar manner across different cortical regions. Moreover, it seems that GABA-related transcript expression is decreased in all aforementioned brain regions to the same extent, so it seems there is no preferential involvement of the DLPFC [27].
Furthermore, the reduced expression of $GABA_A$ receptor $\alpha 1$ and δ subunits in these cortical areas imply that reduced phasic and tonic inhibition, respectively, might be a feature shared by multiple cortical regions.

Furthermore, in addition to the ACC, primary visual cortex and primary motor cortex which demonstrated similar GABAergic expression deficits as the DLPFC, the orbital frontal cortex (OFC), superior temporal gyrus (STG), striatum and thalamus revealed a diminished GAD67 mRNA expression as well [4]. In addition, the STG and auditory gyri demonstrated reduced GAT-1 protein levels [70]. Reduction in GABAergic activity in the OFC could lead to disturbances related to emotional and cognitive functioning. Therefore, the deficit inhibitory activity of the OFC may underlie symptoms regarding social withdrawal and aphetic behavior [74]. In addition, abnormalities in the STG could contribute to deficit auditory processing and auditory hallucinations [4]. These findings imply that the aberrations seen in the DLPFC may not be due to alterations in DLPFC circuitry, but that the altered transcript levels appear to be the consequence of a common upstream mechanism that operates across multiple cortical areas.

Integration of the post-mortem findings on GABAergic neurotransmission

A possible integrative model for the alterations in GABA neurotransmission is that a compromised subset of PFC GABA neurons is present in schizophrenia. In contrast to the reduced GAD67 and the consequent attenuation of inhibitory GABAergic neurotransmission, the reduction of GAT-1 mRNA expression tends to increase the synaptic activity of GABA [52]. In addition, $GABA_A$ receptors are upregulated in postsynaptic pyramidal neurons, which suggests a compensatory increase in response to the decreased extracellular GABA concentrations [58;63;64]. However, based on postmortem studies, it is not possible to identify the initial deficit in the pathological chain and, therefore, two scenarios are possible.

The most likely scenario is an overall reduced GABAergic activity in schizophrenia. This implies that the initial step in this specific pathologic process is the presynaptic reduction of GABA synthesis, followed by a secondary, compensatory reduction of reuptake by means of GAT-1 and by compensatory

upregulation of postsynaptic GABA-receptors [2;75;76]. This synergetic attempt, to improve the GABAergic neurotransmission at the synapse of the pyramidal neuron AIS, serves to compensate for the initial deficit in synthesis of GABA. Consistent with the theory that the reduction of synthesis is the first step in the pathological chain, mice lacking the GAT-1 gene do not develop diminished levels of GAD67 mRNA. This indicates that the reduction of GAD67 is the initial event [77]. Furthermore, GABA hypofunction due to decreased synthesis reflected by the diminished levels of GAD67 mRNA was imitated in rats by means of pharmacological blockade of prefrontal $GABA_A$ receptors. This resulted in impaired working memory performance, a cognitive function characteristically disturbed in patients with schizophrenia [78;79]. However, it is still controversial whether the compensatory mechanisms are sufficient to overcome the decreased GABA synthesis. In other words, it is unknown if the net effect of the diminished presynaptic synthesis on the one hand and the decreased re-uptake increased postsynaptic reception on the other hand result in an increase or decrease of the inhibitory tone on pyramidal cells by GABA-ergic neurons [52]. In conclusion, the most likely scenario is that reduced presynaptic GABA production results in a reduced reuptake of GABA and in upregulated postsynaptic GABA-receptors in schizophrenia.

Alternatively, an excessive increase of GABAergic activity due to both primary diminished reuptake as upregulated post-synaptic receptors may also be an initial step in the pathological process followed by secondary compensatory downregulation of GAD67 mRNA in chandelier neurons due to the excessive GABAergic activity. Furthermore, the effects of pharmaceuticals involved in GABAergic neurotransmission seem to be in line with the hypothesis of excess GABAergic activity. For example, lorazepam, a positive allosteric modulator of GABAergic neurotransmission, results in a deterioration of working memory aberrations while flumazenil, a partial inverse agonist, leads to improvement of the working memory deficits [52]. Thus, according to this scenario, excessive GABAergic activity could be the result of an initial post-synaptic upregulation of the $GABA_A$ receptor and downregulation of the presynaptic GABA reuptake transporters as a first step in the pathological chain [52].

Finally, the aberrations seen in the DLPFC may not be due to alterations in DLPFC circuitry, but instead reflect transcript levels that are a consequence of a common upstream mechanism that operates across multiple cortical areas in schizophrenia.

In conclusion, the most likely scenario involves reduced GABA concentrations in due to a compromised production of GABA reflected by the diminished

concentration of GAD67 mRNA. However due to the observation that presynaptic GAT-1 is reduced and postsynaptic receptors are upregulated, post-mortem studies do not provide a conclusive answer about the net GABAergic concentrations and activity. Therefore, in-vivo studies could provide additional insights into GABA levels in clinical states contributing to a more definitive formulation about the pathological cascade and GABAergic alterations in schizophrenia

Proton MRS

Proton magnetic resonance spectroscopy (^{1}H-MRS) enables in vivo measurement of multiple metabolites among which N-acetylaspartate (NAA), creatine choline, glutamate and glutamine. Quantification of GABA is rather challenging because of two reasons. Firstly, in comparison with other metabolites, the concentration of GABA is low resulting in a low signal-to-noise ratio [80-82]. Secondly, the GABA signal is overlapped by signals of higher intensity [16;83]. Because of this overlap other metabolite signals, the GABA signal can only be reliably measured using spectral editing techniques.

Based on presynaptic and postsynaptic GABAergic alterations in post-mortem studies, it is possible to identify numerous brain areas such as the ACC, primary visual cortex, primary motor cortex, orbital frontal cortex, basal ganglia, superior temporal gyrus, thalamus, but especially the DLPFC in which it is expected to measure altered GABAergic concentrations by ^{1}H-MIRS. As mentioned before, post-mortem studies do not provide a conclusive answer about the net GABAergic concentrations and activity. Therefore ^{1}H-MRS could provide additional insights contributing to a more definitive formulation about the pathological cascade and GABAergic alterations in schizophrenia. However, up until now MRS studies on GABA in schizophrenia are rather scarce. Moreover, the current literature is inconsistent regarding the measured GABA levels in different brain regions of patients with schizophrenia. Currently, two studies reported GABA reductions [84;85], two studies reported unchanged GABA levels [86;87], and two studies reported increased levels (**Table 4**) [88;89].

The fluctuating and inconsistent findings of the few MRS studies that have been published so far in schizophrenia could be explained by several factors such as small and heterogeneous sample sizes, low magnetic field strengths resulting in inaccurate measurements of GABA spectral peaks, methodological limitations leading to relatively large voxel volumes and marginal adjustments with regard to gray and white matter differences [16]. Moreover, most studies measured GABA

Table 4: Proton magnetic resonance spectroscopy measures of GABA concentration in schizophrenia

Author	Subjects [I]	Study	Medication	Results chronic	Results early stage	Mixed population [II]
Goto et al.[84]	18 first episode 18 controls	3T MRI MEGA-PRESS Voxel 3.0cm x 3.0cm x 3.0cm	Atypical AP at baseline [III]	-	- GABA/creatine (Cr) striatum ↓ - frontal lobe ~ - parieto-occipital lobe ~	-
Ongur et al.[88]	20 chronic, 1 first episode 19 controls	4T MRI MEGA-PRESS 2.3cm x 2.2cm x 3.3cm	Stable (unknown) medication regimen	- GABA/creatinine ACC ↑ - GABA/ creatinineparieto-occipital cortex ↑[IV]	-	-
Tayoshi et al.[86]	38 chronic 29 controls	3T MRI MEGA-PRESS Voxel 3.0cm x 3.0cm x 3.0cm	16 typical +/- atypical 22 atypical [V]	- GABA ACC ~ - GABA left basal ganglia (ltBG) ~	-	-
Yoon et al. [85]	13 chronic + first episode 13 controls	3T MRI MEGA-PRESS 3.5cm x 3.0cm x 2.5cm	5 unmedicated [VI] 1 typical and 7 atypical AP	-	-	- GABA occipital cortex ↓
Kegeles et al.[89]	32 chronic 22 controls	3T MRI MEGA-PRESS Voxel: 2.5cm x 3.0cm x 2.5cm	16 unmedicated [VII] 16 atypical antipsychotics	- GABA and Glx DLPFC ~ - GABA and Glx MPFC ↑ - Medicated patients: GABA and Glx in DLPFC and MPFC ~	-	-
Rowland et al.[87]	21 chronic 20 controls	3T MRI MEGA-PRESS Voxel: 3.5cm x 3.5cm x 3.5cm	2 typical antipsychotics 19 atypical antipsychotics [VIII]	- GABA ACC ~ [IX] - GABA centrum semiovale (CSO) ~ - Glx ACC and CSO ↓	-	-

[I] Mean duration of illness of patients with schizophrenia per study: Goto et al.: scans within six months after the symptoms emerged; Ongur et al.: mean duration of illness unknown; Tayoshi et al.: mean duration of illness 11.1±9.4 years; Yoon et al.: mean duration of illness unknown; Kegeles et al.: mean duration of illness for the un-medicated group 7±7 years, for the medicated group 9±8 years; Rowland et al.: mean duration of illness 7.7±4.1 years for the younger group, for the older group 25.5±6.5 years.

[II] Mixture of chronic and first episode (FE) patient

[III] Chlorpromazine equivalent, total cumulative exposure was 9381 ± 1382mg;

[IV] Study population consisted of 20 chronic patients and 1 first episode patient

[V] Chlorpromazine equivalent, mean dose of antipsychotics 423.7 ± 362.3 mg

[VI] Five unmedicated for at least a month at the time of testing, four of which never received treatment for more than two weeks. The mean dose of chlorpromazine equivalent for the medicated patients is 433 ± 228mg.

[VII] Seven unmedicated defined as no treatment for a minimum of fourteen days prior to the moment of testing and nine antipsychotic medication naïve. Antipsychotic dose is unknown for medicated patients, but has not been altered during four weeks prior to the scan.

[VIII] Dose of antipsychotics unknown, but not altered during three months prior to the scan

[IX] GABA levels were higher in younger than older patients.

referenced to creatine and although this method is the common approach at the moment, fluctuations in creatine concentrations could be, to a certain extent, responsible for the observed GABAergic findings. However, the most prominent limitation compromises the undetermined role of antipsychotic medication use with regard to GABA levels measured by ^{1}H-MRS.

MRS-GABA and antipsychotic medication

In thirty-eight chronic schizophrenia patients, higher GABA concentrations were found in the left basal ganglia in patients using typical antipsychotics as compared to patients using atypical antipsychotics. Furthermore, a positive correlation was reported between GABA concentration in the left basal ganglia and anticholinergic medication. It is thus possible that antipsychotic medication influences GABA concentrations and different types of medications could have differing effects [86].

However, in patients diagnosed with schizophrenia and using antipsychotic medication at baseline, the use of atypical antipsychotics did not have any effects on GABA concentrations in the left basal ganglia, frontal lobe and parieto-occipital lobe during a follow-up period of six months [90]. At baseline, the concentration of GABA in the left basal ganglia in these first-episode patients was decreased 84, but this reduction was not reversed after six months of treatment with antipsychotic medication [90]. Interestingly, clinical condition, assessed by PANSS-scores, did improve during this time period. This suggests that medication use has no profound effect on GABA concentrations in patients with schizophrenia although there does occur a clinical improvement [84;90]. However, it is also possible that the medication regimen prevented further progressive reduction of GABA concentrations in these patients. Studying patients not taking antipsychotic medication may provide valuable additional insights regarding this matter. A recent study addressed this topic and evaluated GABA concentration in sixteen un-medicated patients, consisting of nine medication naïve patients and seven patients with no antipsychotic medication use fourteen days prior to the investigation. This study observed higher GABA concentrations in never and un-medicated patients compared to medicated patients [89]. This implies that medication use might lead to a normalization of GABA concentrations [89]. However, as mentioned before, medicated patients did not show any alterations regarding GABA concentrations after six months of antipsychotic therapy [90]. Possibly, patients that were minimally treated at baseline [90] differed from those that were medication naïve [89;90] and the normalization of GABA concentrations due to antipsychotic treatment takes place at the beginning of the treatment. To formulate a conclusive answer, future

studies are required which assess both within-subject medication and medication naïve study designs. In conclusion, many factors contribute to the inconsistency in literature and future studies need to take these factors into account to reconcile the fluctuating findings.

at baseline [90] differed from those that were medication naïve [89;90]. To formulate a conclusive answer, future studies are required which assess both within-subject medication and medication naïve study designs. In conclusion, many factors contribute to the inconsistency in literature and future studies need to take these factors into account to reconcile the fluctuating findings.

MRS-GABA and cognition

The observed GABAergic neurotransmission alterations may have a functional significance. GABA measurement in the visual cortex revealed reduced concentrations and this decrease was positively correlated with orientation-specific surround suppression (OSSS). OSSS is a behavioral measure of visual inhibition and it is believed that this process relies on GABAergic neurotransmission in the visual cortex 85. Furthermore, patients with schizophrenia which showed poorer performance on attention tests were correlated with decreased GABA concentrations 87. These observations are consistent with the GABA deficit hypothesis which states that reduced GABAergic neurotransmission results in cognitive deficits and these studies also imply that measurement of GABA in a pool of cortical GABA neurons has a direct functional relationship with GABA mediated functioning 16. Since the GABAergic expression deficits exhibit a pancortical involvement, it is likely that such aberrations generalize to other cortical areas 27;85.has a direct functional relationship with GABA mediated functioning [16]. Since the GABAergic expression deficits exhibit a pancortical involvement, it is likely that such aberrations generalize to other cortical areas [27;85].

Integrating MRS-GABA with post-mortem GABA findings

The reported elevation of GABA levels in the MPFC in unmedicated patients seem to be inconsistent with the results of post-mortem studies which exhibit an impaired GABA synthesis of parvalbumin-containing subclasses of GABA neurons reflected by diminished GAD67 mRNA levels [89]. However, this discrepancy could be explained by the extensive exposure of the post-mortem brain samples to antipsychotic medication in predominantly chronically ill patients. In addition, one study reported

higher GABA levels in younger patients than older patients with schizophrenia and illness course may therefore affect GABA concentrations [87]. Consistent with this thought, some post-mortem studies did find unaltered GAD67 mRNA levels in sample sizes which were significantly older compared to the majority of studies which observed decreased GAD67 mRNA levels [33]. Furthermore, the observed elevated GABA levels in the MPFC by ^{1}H-MRS might also be an overcompensation of other subgroups of GABA neurons [89]. The NMDA-receptor hypofunction hypothesis puts forward that an intrinsic deficit of GABA neurons including impaired GABA synthesis results in disinhibition of pyramidal neurons. The deficit regulation of pyramidal neurons by GABA-ergic neurotransmission leads to glutamate elevations [91;92]. Therefore, the remaining unimpaired subclasses (other than the parvalbumin-containing subclass) could be stimulated by the increased glutamergic activity and this could serve as a compensation for the diminished synthesis in the parvalbumin-containing subclass [89].

Recent advantages in ultrahigh-field MR techniques allow for better assessment of GABA concentrations and future studies must point out whether in vivo measurement of GABA corresponds with the observed GABA deficiency in postmortem tissues and whether the GABAergic deficits occur in a pan-cortical manner. Moreover, futures studies might point out if GABA concentrations predict functional outcome and if alterations in GABA concentrations relate to therapy response. It is clear that GABA measurement by in vivo MR spectroscopy could be of great value, but it is also evident that further work is needed to provide additional information on the validation of MR spectroscopy of GABA in schizophrenia.

Conclusion

Converging evidence implicate alterations in both presynaptic and postsynaptic components of GABA neurotransmission to fulfill an important role in the pathophysiology of schizophrenia. Multiple laboratories using either in situ hybridization, DNA microarray or real-time quantitative PCR have consistently found reduced levels of GAD67 mRNA or a reduced density of neurons positive for GAD67 mRNA in the DLPFC as one of the most consistent findings with regard to pathological changes in schizophrenia. This decrease is the consequence of a reduction of GAD67 mRNA in a subset of GABA neurons. The affected neurons appear to include the parvalbumin-containing neurons. Parvalbumin-positive cells in the DLPFC include chandelier cells, targeting the upregulated α 2 receptor subunit at the axon initial segment of the pyramidal neuron. Furthermore, since

GAD67 mRNA expression deficits were also observed in layers without parvalbumin expression, other sublclasses may attribute to the observed GABAergic gene expression deficits as well. Furthermore, since other brain regions demonstrated similar GABAergic gene expression deficits as the DLPFC, disturbances in GABAergic neurotransmission could be the consequence of a common upstream effect. Therefore, identifying a common pathophysiology might give rise to new pharmacological opportunities in the treatment of schizophrenia. Measurement of GABA levels *in vivo* by means of ultra-high field MRS offers the possibility to approach the illness from a unique perspective and provides additional insights in the relationship between deficit components of GABA neurotransmission and GABA-mediated inhibitory activity. However, the current literature is inconsistent regarding the measured GABA levels in different brain regions of patients with schizophrenia. Future MRS studies using GABA editing are required in order to give us a better understanding of the pathophysiology of schizophrenia.

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4

Glutamate changes in healthy young adulthood

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European Neuropsychopharmacology 2012; Epub ahead of print

Abstract

Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system and has been associated with several cognitive functions that are known to change with age. In rodents and humans age-related glutamate changes have been found in several brain areas. In this cross-sectional study the presence and extent of age-associated glutamate changes in the medial frontal cortex of healthy young adults were measured. Proton magnetic resonance spectroscopy (^{1}H-MRS) and brain imaging were performed at 7 tesla in a 2x2x2 cm^3 voxel in 33 participants between 18 and 31 years old. Glutamate concentrations and gray and white matter volume could be successfully determined at an ultra-high magnetic field strength. Glutamate concentrations were lower in older individuals (0.33 mM/year). This decline is in line with gray matter thinning in the medial frontal cortex, but could not be explained by cortical thinning alone. Therefore, the decrease in glutamate in young adulthood may be due to physiological changes rather than anatomical changes.

Introduction

Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system and principal neurotransmitter of cortical efferent systems [1]. Glutamate is involved in functions such as learning and memory [2], emotion and motivation [3;4] and motor behaviour [5]. The primary location of glutamate is in neurons and astrocytes. Only a small fraction of the total glutamate pool is present in the extracellular space. Glutamate is continuously released from cells and removed from the synaptic clefts and extracellular space. This removal is essential since exposure to high extracellular glutamate concentrations can have neurotoxic effects [6;7]. This neurotoxicity has been implicated in neurodegenerative diseases like multiple sclerosis [8], amyotrophic lateral sclerosis [9], Alzheimer's dementia [10], Parkinson's disease [11] and schizophrenia [12]. Recently, meta-analysis showed a progressive decrease with age of glutamate concentrations in the frontal brain region in schizophrenia [13], suggesting that too low levels of intra or extracellular glutamate may also be associated with neuropsychiatric diseases. Studying glutamate levels in normal maturation may contribute to elucidating the role of glutamate in cognitive decline in health and in neurological and neuropsychiatric disorders.

Studies in rodents as well as in humans have shown that age-related glutamate decreases occur in several brain areas, including motor areas, sensory areas and the hippocampus [14-16]. Using these methods, in the rodent frontal cortex decreases in glutamate with age as well as neuronal loss have been found [17-19]. Since glutamate is present in most cortical neurons, the observed decreases in glutamate may be associated with the age-dependent neuronal pruning [17]. In humans, the frontal cortex shows thinning with increasing age, which could be due to shrinkage of neurons as well as neuronal loss [20;21], and might possibly be correlated with decreases in glutamate concentrations. Structural brain changes in the frontal cortex in young adulthood [22] suggest that there are physiological changes going on during young adulthood. However, at this point in time the physiology underlying these structural brain changes is not well understood.

In this study we measured glutamate concentrations in the medial frontal brain region of healthy, young adult subjects, using high-field magnetic resonance spectroscopy. Proton magnetic resonance spectroscopy (^{1}H-MRS) can be used to non-invasively measure metabolite concentrations *in vivo*. Until now, studies investigating glutamate during aging using ^{1}H-MRS were conducted at field strengths of 4 tesla or lower. Previous studies showed a decrease of glutamate with age in the mesial motor cortex between 26 and 54 years [15] and dorsolateral

prefrontal cortex between 19 and 31 years [23;24], a trend of an age-related glutamate decline in frontal white matter between 21 and 71 years [25], and age-related increase of glutamate in the orbitofrontal cortex between 19 and 52 years [26]. Since measurement of glutamate with ^{1}H-MRS is challenging at lower field strengths, due to its spectral overlap with glutamine, a magnetic field strength of 7 tesla was used in this study to accurately determine glutamate concentrations in the human brain. In addition, we measured N-acetyl aspartate (NAA), a marker of neuronal integrity, creatine, a marker of energy metabolism, and choline, a marker of membrane turnover [27]. Also, most of the previous studies compared a younger age group with an older age group, thereby not investigating the alterations occurring within each age group. We measured glutamate, NAA, creatine and choline, and the fractions of gray and white matter in the medial frontal cortex, which is an area that decreases in volume in young adulthood [20], and has been reported to be decreased in schizophrenia [28]. With this study, we investigate the relation between glutamate levels in the frontal cortex and age within a group of young adults.

Methods

Subjects

37 healthy subjects (19 men and 18 women) participated in this study, after giving written informed consent as approved by the Medical Ethics Committee of the University Medical Center Utrecht, The Netherlands. Participants were between 18.3 and 31.5 years old (mean $\pm$ SD = 24.8 $\pm$ 3.4 years). Participants had no major psychiatric or neurological history and no history of drug or alcohol abuse, as tested with the Mini International Neuropsychiatric Interview Plus (MINI-Plus) [29]. Participants had no first-degree relatives with psychiatric or neurological disorders. Four volunteers were excluded from the study due to poor quality of MR spectra or the T1-weighted image. Hence, the results of this study are based on 33 subjects.

MRS acquisition

^{1}H-MRS experiments were performed on a 7 tesla whole body MR scanner (Philips, Cleveland, USA) with a birdcage transmit head coil (Nova Medical, Inc., Burlington, MA, USA) in combination with a 16-channel receive coil (Nova Medical, Inc., Burlington, MA, USA). First, a T1-weighted MP-RAGE sequence (450 slices, slice thickness = 0.8 mm, TR = 7 ms, TE = 3 ms, flip angle = 8 degrees, FOV = 250x200x180 mm^{3}, 312x312 acquisition matrix, SENSE factor 2.7, scan duration

= 408 s) was obtained for anatomical reference and gray and white matter tissue classification.

After obtaining the anatomical image, MR spectra were acquired of the medial frontal brain region (**Figure 1**). For this, a stimulated echo acquisition mode (STEAM) sequence (TR = 2000 ms, TE = 7 ms, TM = 11 ms, single voxel, voxel size = 2x2x2 cm^3, B_1 = 20 μT) was used, placing the voxel of interest in the medial frontal cortex, in front of the genu of the corpus callosum. Water suppression was applied (SWAMP [30]) for obtaining the MR spectrum with glutamate, NAA, creatine and choline (acquisition time = 260s). An MR spectrum without water suppression was obtained for quantification (carrier frequency was set to the chemical shift of H_2O, acquisition time = 10s). Second order B_0 shimming was based on the FASTERMAP algorithm [31;32]. After selecting the volume of interest, local B_1 field was calibrated [33]. Besides glutamate, we also quantified NAA, creatine and choline.

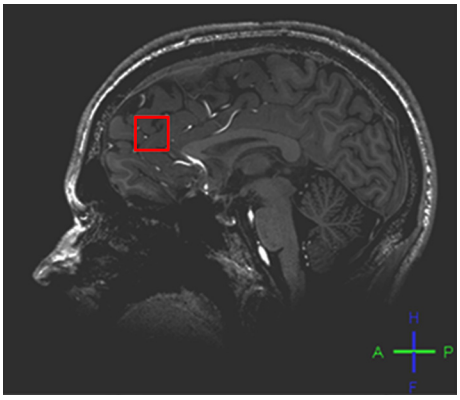

Figure 1: Voxel placement. The voxel (2x2x2 cm3) was placed in the medial frontal cortex in front of the genu of the corpus callosum.

Spectral fitting and quantification

Spectral fitting was performed using the Accurate Quantitation of Short Echo time domain Signals (AQSES) algorithm, implemented in the Simulation Package based on In vitro Databases (SPID) [34]. Metabolite levels were estimated using the water signal as an internal reference. The concentration of each metabolite was calculated as follows:

[1]

$$[\mathrm{met}] = \left(\frac{\frac{\mathrm{signal}_{\mathrm{met}}}{\mathrm{signal}_{\mathrm{water}}} * \left(\mathrm{volGM} * [\mathrm{water}_{\mathrm{GM}}] + \mathrm{volWM} * [\mathrm{water}_{\mathrm{WM}}] + \mathrm{volCSF} * [\mathrm{water}_{\mathrm{pure}}] \right)}{\mathrm{volGM} + \mathrm{volWM}} \right)$$

Where [met] is the metabolite concentration, signal $_{met}$ is the area under the curve of the metabolite spectrum and signal $_{water}$ is the area under the curve of the water spectrum; volGM, volWM and volCSF are respectively the gray matter content, white matter content and cerebrospinal fluid (CSF) content in the voxel; and [water$_{GM}$], [water$_{WM}$] and [water$_{pure}$] are respectively the water concentration in gray matter, white matter or CSF. The segmentation of the anatomical image in gray matter, white matter and CSF fractions was done using the software package SPM8 (http://www.fil.ion.ucl.ac.uk/spm/software/spm8). After alignment of the ^{1}H-MRS voxel with the anatomical scan, the gray matter, white matter and CSF fractions in the ^{1}H-MRS voxel was determined.

Statistical analysis

For regression analyses, the general linear model (GLM) was applied using the statistical environment R [35], with metabolite concentration as the dependent variable and age, gender and gray matter fraction as independent variables. Gray matter fraction was calculated as follows:

[2]

$$GMfraction = \frac{volGM}{volGM + volWM}$$

Where GMfraction is the proportion of gray matter to total brain tissue in the ^{1}H-MRS voxel, GM is the proportion of gray matter in the ^{1}H-MRS voxel, and WM is the proportion of white matter in the ^{1}H-MRS voxel.

Results

The glutamate resonance at 2.3 ppm was well resolved and could be obtained with a high signal to noise ratio and good spectral resolution as revealed by visual inspection of the spectrum (**Figure 2**).

The glutamate concentration in the medial frontal cortex changed significantly with age (b=-0.33, t=-2.5, df=30, p=0.02; mean $\pm$ SD = 6.1 $\pm$ 2.7) (Figure 3). No significant effects of gray matter fraction (b=2.74, t=1.2, p=0.23) and gender (b=-0.42, t=0.5, p=0.65) were found.

NAA did not show any significant age-related changes (b=-0.1, t=1.3, p=0.22; mean $\pm$ SD = 6.2 $\pm$ 1.7) (Figure 4). NAA did not have a significant association with the gray matter fraction in the voxel (b=2.14, t=1.4, p=0.16).

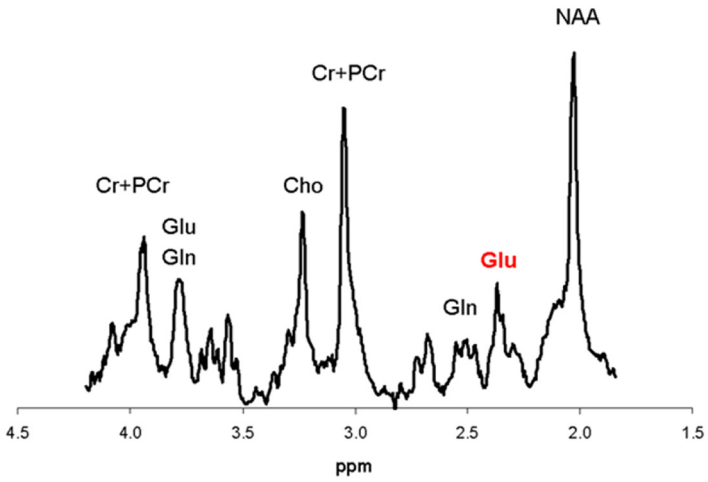

Figure 2: A typical spectrum, as measured in this study using the stimulated echo acquisition mode (STEAM) at a magnetic field strength of 7 tesla in a 2x2x2 cm3 voxel placed in the medial frontal cortex.(Cr=creatine, PCr=phosphocreatine, Cho=choline, Gln=glutamine, Glu=glutamate, NAA=N-acetyl aspartate).

Creatine concentrations changed significantly with age (b=-0.22, t=-3.3, p=0.002; mean ± SD = 6.0 ± 1.4) (Figure 5). Creatine did not have significant associations with the gray matter fraction in the voxel (b=2.05, t=1.8, p=0.08) and gender (b=0.68, t=1.5, p=0.15).

Choline concentrations show an age-related decrease, however, this is not significant (b=-0.28, t=-1.9, p=0.06; mean ± SD = 1.5 ± 0.4) (Figure 6). Choline did not have significant associations with the gray matter fraction in the voxel (b=0.44, t=2.4, p=0.86) and gender (b=0.80, t=0.8, p=0.44).

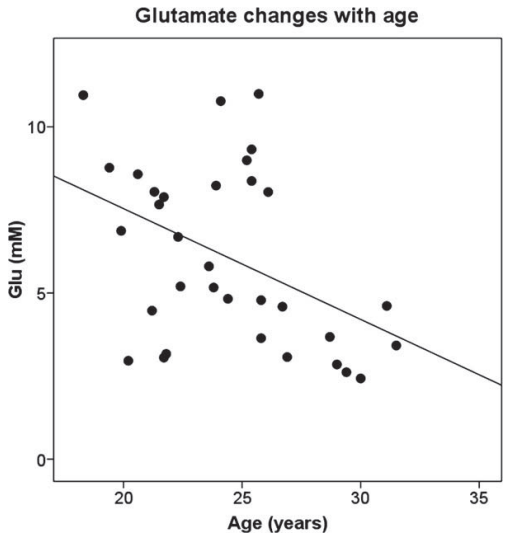

Figure 3: Linear regression analysis of age on glutamate concentrations in the medial frontal cortex (b=-0.33, t=-2.5, p=0.02).

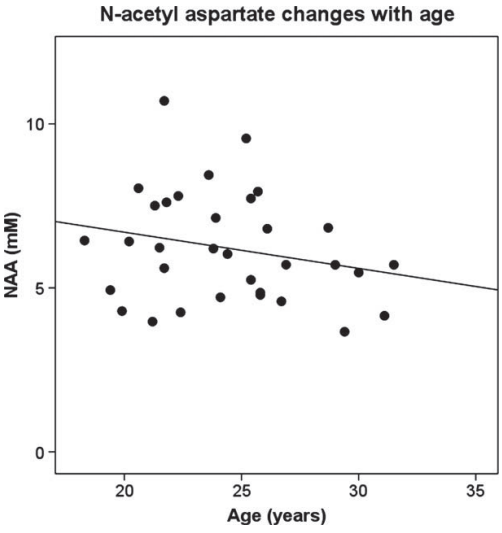

Figure 4: Linear regression analysis of age on NAA concentrations in the medial frontal region (b=0.07, t=1.1, p=0.28).

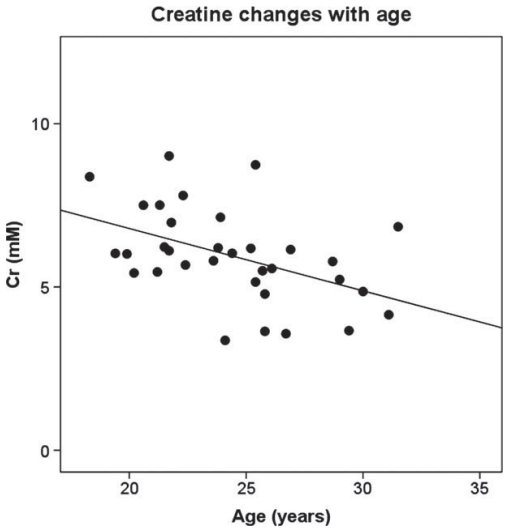

Figure 5: Linear regression analysis of age on creatine concentrations in the medial frontal region (b=-0.22, t=-3.3, p=0.002).

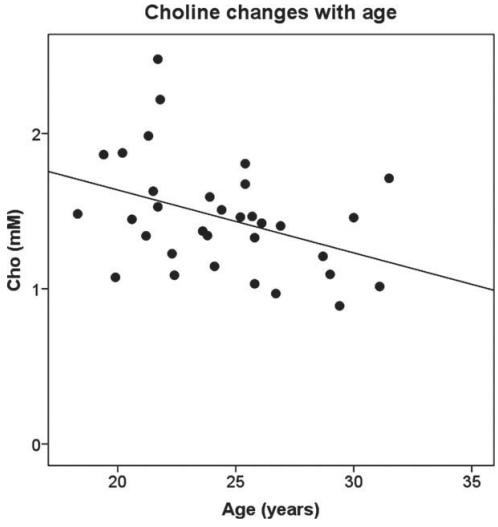

Figure 6: Linear regression analysis of age on choline concentrations in the medial frontal region (b=-0.28, t=-1.9, p=0.06).

Discussion

The main finding of this study is a decrease in glutamate concentration with age in a relatively small age range of 18 to 31 years in medial frontal cortex of the human brain. Concentrations of NAA, a marker of neuronal integrity, did not show any significant changes in this age range, which indicates that there are no alterations in brain tissue or neuronal function in this brain region in young adult individuals. This finding is further strengthened by the fact that gray matter volume did not reveal a significant influence on the age-related decline in glutamate in the medial frontal cortex.

In healthy aging, the ^{1}H-MRS profile of the human brain shows region-specific alterations, the largest alterations occurring in (pre)frontal brain regions and in particular in glutamate and GABA concentrations [23]. Indeed, our findings corroborate that glutamate concentrations decrease with age in the human frontal cortex, already at a young adult age. In a meta-analysis of longitudinal MRI-studies of whole brain volume change in healthy individuals it was shown that there is a period of stable brain volume between 18 and 35 years of age [22], which supports the suggestion that the glutamate decline observed in this period has a physiological background and is not primarily a reflection of changes in brain volume. The alterations in glutamate concentration in the medial prefrontal cortex may precede changes in cortical volume or may reflect the consequence of cortical changes that occur during adolescence. During the transition from adolescence to adulthood synaptic density decreases and (mainly) glutamatergic synapses are eliminated, reflecting synaptic pruning [36-38]. Interestingly, both glutamate levels [2;17;39] and efficiency of structural and functional brain networks [40-43] are associated with cognitive functioning. Thus, possibly the glutamate decline in young adulthood may reflect brain maturation.

The glutamate signal that is detected in the human brain by ^{1}H-MRS does not distinguish between glutamate used in metabolic pathways and glutamate used for neurotransmission. Since the majority of glutamate is present in the metabolic pool [44], the decreases in glutamate are possibly due to changes in metabolic activity [17;45]. This may reflect a decreased neuronal integrity since glutamate is primarily located in neurons. However, we did not find any significant age-related decline in the concentrations of NAA, which implies that it is unlikely that the decrease with age in glutamate concentration in the prefrontal cortex in these young adult humans represents changes in neuronal integrity. Indeed, it is more likely that the age-related decline in glutamate concentrations at this age point to changes in glutamatergic neurotransmission and neuronal metabolism. Changes in the

regulation of glutaminase, the enzyme that converts glutamine into glutamate, are present in the aging brain [46;47], which could lead to a reduction of the glutamine-glutamate cycle flux [48]. A decrease with age in the density of the NMDA-type of glutamate receptor has also been found [49], possibly resulting in reduced uptake capacity [17]. The age-related decline in glutamate could in turn result in reduced glutamate-receptor activation, in particular of the NMDA-type, which may lead to cognitive deficits [50;51]. However, since there are no signs of cognitive alterations in young adulthood, and yet there are signs of synaptic pruning , the decrease in glutamate might alternatively suggest a higher efficiency of glutamate signalling in this period. The latter may possibly be a result of maturation of neural systems [52]. In fact, the age-related decreases that were found for concentrations of creatine, a marker of energy metabolism, and choline, a marker of membrane turnover, support this notion.

The development of the glutamatergic neurotransmitter system during healthy maturation may also provide insight in pathologies underlying neurodegenerative diseases, like schizophrenia. Glutamate concentrations decrease progressively with age in patients with schizophrenia as compared with healthy individuals [13]. Since the results of this study show that glutamate concentrations in healthy individuals are declining with age as well, this implicates that glutamate levels in patients with schizophrenia decline at an even faster rate.

There are some points that have to be taken into consideration when interpreting its findings. The ^{1}H-MRS technique that was used in this study, STEAM, has the advantage of combining a short TE with a long TR, thereby reducing quantification errors due to T_1 and T_2 relaxations, which are possibly age-dependent [16;53]. However, STEAM suffers from signal to noise ratio (SNR) loss and only half of the available signal can be obtained, therefore, the application of newly developed ^{1}H-MRS sequences for 7T might result in a better SNR and therefore more sensitive measures in the future [54]. Also, the use of water as an internal reference to calculate metabolite concentrations might induce quantification errors, since a possible decrease of water concentrations with age is not considered [15;55]. However, between 20 and 70 years of age, water concentrations in the medial frontal lobe do not show any significant changes [56]. In this study, only a small age range was investigated. It would be interesting to examine glutamate levels at older ages as well, thus acquiring a complete profile of glutamate changes throughout adult life, which could help elucidate the physiology behind several neural processes that show age-related alterations.

In conclusion, this study shows a decline in glutamate concentrations in the medial frontal cortex in healthy young adulthood and no alterations in markers of neuronal integrity, suggesting there are physiological changes occurring in this age range.

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5

Detection of glutamate alterations in the human brain using ^{1}H-MRS with sLASER at 7T

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Abstract

The purpose of this study is To assess reproducibility of glutamate measurement in the human brain by two short TE ^{1}H-MRS sequences (STEAM and sLASER) at 7T. Reliable assessment of glutamate is important when studying a variety of neurological and neuropsychiatric disorders. At 7T, the glutamate signal can be separated from the glutamine signal and hence more accurately measured as compared to lower field strengths. A sLASER sequence has been developed for 7T, using field focusing at short TE resulting in twice as much signal as can be obtained using STEAM, and improved localization accuracy due to a decreased chemical shift artifact.

Eight subjects were scanned twice using both STEAM and sLASER. Data was acquired from the frontal and occipital brain region. Subsequently, intraclass correlations (ICC) were computed for the estimated metabolite concentrations. sLASER has higher ICC's for glutamate concentration as compared to STEAM in both the frontal and occipital VOI, which is probably due to the higher sensitivity and localization accuracy. We conclude that sLASER ^{1}H-MRS at 7T is a reliable method to obtain reproducible measures of glutamate levels in the human brain at such high accuracy that individual variability, even between age-matched subjects, is measured.

Introduction

In vivo ^{1}H magnetic resonance spectroscopy (^{1}H-MRS) can be used to determine glutamate (Glu) levels in the human brain. Glutamate is the primary excitatory neurotransmitter in the mammalian central nervous system. Examining glutamate levels is important when studying a variety of neuropsychiatric conditions, including schizophrenia, bipolar disorder, depression, Alzheimer's dementia, and anxiety disorders [1]. Up until now, the majority of studies examining glutamate in psychiatric disorders using ^{1}H-MRS were conducted at magnetic field strengths of 4 T or lower. However, measurement of glutamate with ^{1}H-MRS is challenging at lower field strengths, due to its spectral overlap with glutamine. A magnetic field strength of 7T results in an increased signal-to-noise ratio (SNR) and chemical shift dispersion, therefore the glutamate and glutamine resonances can be adequately separated, and glutamate can be accurately determined [2]. The full in-phase glutamate signal can be acquired using short echo time (TE) ^{1}H-MRS sequences. For human brain applications, the STEAM (stimulated echo acquisition mode) sequence is the most commonly used method for localization [3]. However, STEAM suffers from severe SNR loss because only half of the available signal can be obtained (**Figure 1**).

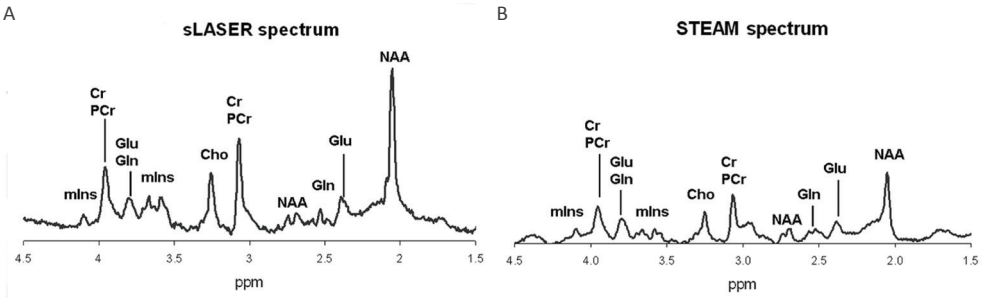

Figure 1: Typical spectra for the sLASER sequence (left) and STEAM sequence (right).

Recently, a sLASER (semi-localized by adiabatic selective refocusing) sequence has been developed for application at a magnetic field strength of 7T. sLASER can be applied to the human brain with a conventional volume head coil, and the full signal can be acquired at short TE with a small chemical shift displacement artefact. The two transmit channels in a conventional volume head coil are driven independently, generating a maximized B_1^+ field and allowing the use of short adiabatic refocusing pulses for single-voxel MRS in most of the brain [4].

When performing clinical ^{1}H-MRS studies, it is necessary to reliably detect changes in metabolite levels caused by diseases. As mentioned above, glutamate assessment in the human brain has been challenging at low magnetic field

strengths due to poor spectral resolution and SNR loss. With the introduction of the sLASER sequence at 7T, these issues could be overcome. To determine if sLASER can be used as a standard in ^{1}H-MRS studies of glutamate levels in psychiatric disorders, it is needed to assess the reproducibility of glutamate measurement with the sLASER sequence. To evaluate this, experiments were performed at two time-points in eight healthy, age-matched volunteers. Both the STEAM and sLASER sequence were used to measure metabolite concentrations in the frontal as well as the occipital brain region. In addition, we fitted the data into metabolite concentrations using three models, each including a different amount of metabolite basis sets, to evaluate the robustness of the results.

Methods

Subjects

Eight healthy subjects (21-29 yr, mean $\pm$ SD = 23.9 $\pm$ 2.4 yr, 3 males, 5 females) were scanned twice, with two weeks between the measurements. Written informed consent, as approved by the institutional ethics board, was given by all volunteers prior to the examinations. Participants had no major psychiatric or neurological history and no history of drug or alcohol abuse, as tested with the Mini International Neuropsychiatric Interview Plus (MINI-Plus) [5]. Participants had no first degree relatives with psychiatric or neurological disorders. We excluded the first measurement with the sLASER sequence in the occipital lobe in one subject because of low spectral quality.

MR acquisition

All investigations were performed on a 7T whole body MR scanner (Philips, Cleveland, OH, USA). A birdcage transmit head coil was used in dual transmit driven by 2x4 kW amplifiers, in combination with a 16-channel receive coil (both Nova Medical, Inc., Burlington, MA, USA). A T_1-weighted MP-RAGE sequence (450 slices, slice thickness = 0.8 mm, TR = 7 ms, TE = 3 ms, flip angle = 8 degrees, FOV = 250 x 200 x 180 mm, 312 x 312 acquisition matrix, SENSE factor 2.7, scan duration = 408 s) was obtained for anatomical reference and gray and white matter tissue classification. ^{1}H-MRS experiments were conducted with two short TE sequences, i.e. STEAM (stimulated echo acquisition mode; TE = 7.8 ms, 128 averages, TR = 2 s) and sLASER (semi-localized by adiabatic selective refocusing; TE = 28 ms, 16 averages, TR = 5 s). Voxels (2 x 2 x 2 cm^3) were located in the left frontal and

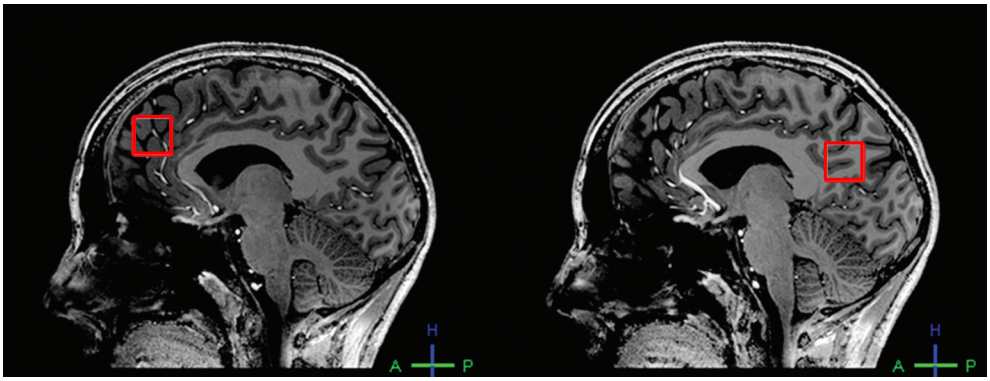

Figure 2: Placement of the frontal (left) and occipital (right) voxel.

left occipital lobe (**Figure 2**). Non water suppressed spectra were obtained for quantification (carrier frequency was set to the chemical shift of H_2O, acquisition time = 10 s). Prior to the MRS exams, second order B_0 shimming was applied using the FASTERMAP algorithm at the voxel of interest [6;7]. Second, at this location, a high B_1 field was generated to minimize chemical shift displacement artefacts [8]. The highest possible B_1 field was generated by optimizing the phase of both transmit channels to locally assure constructive B_1 interferences [4].

Spectral fitting and quantification

Retrospective phase and frequency alignment was performed on all data sets of each measurement [9]. Spectral fitting was performed with LCModel-based software implemented in Matlab [10], which uses a priori knowledge of the spectral components to fit metabolite resonances [11]. Three separate fitting procedures were performed on all data sets to examine if the amount of metabolites included in the model influences the reproducibility of the data. In the separate fitting procedures 8 (PC, PE, PCr, NAA, Glu, Gln, GSH, mIns), 12 (Cho, PC, GPC, PE, Cr, PCr, NAA, NAAG, Glu, Gln, GSH, mIns) or 16 (Ace, Asp, Cho, PC, GPC, PE, Cr, PCr, NAA, NAAG, GABA, Glu, Gln, GSH, mIns, Tau) metabolites and a measured macromolecular baseline [12] were fitted to the spectra (**Table 1**).

Metabolite levels were estimated using the water signal as an internal reference and calculated as follows:

Where [met] is the metabolite concentration, $signal_{met}$ is the fitted signal

$$[\mathrm{met}] = \left(\frac{\frac{\mathrm{signal}_{\mathrm{met}}}{\mathrm{signal}_{\mathrm{water}}} * \left(\mathrm{volGM} * [\mathrm{water}_{\mathrm{GM}}] + \mathrm{volWM} * [\mathrm{water}_{\mathrm{WM}}] + \mathrm{volCSF} * [\mathrm{water}_{\mathrm{pure}}] \right)}{\mathrm{volGM} + \mathrm{volWM}} \right)$$

intensity of the metabolite, accounting for the number of protons, and $signal_{water}$ is the fitted signal intensity of water, accounting for the number of protons; volGM, volWM and volCSF are respectively the gray matter content, white matter content and cerebrospinal fluid (CSF) content in the voxel; and [$water_{GM}$], [$water_{WM}$], and [$water_{pure}$] are respectively the water concentration in gray matter, white matter or CSF. For determining the contribution of gray matter, white matter and CSF of each voxel, the software package SPM8 was used to segment the T1-weighted image. In the T_1-weighted image, the position of the ^{1}H-MRS voxel was determined, after which the amount of gray matter, white matter and CSF in the ^{1}H-MRS voxel was computed.

Table 1: Metabolites that were fitted to the spectra using three different fitting procedures

Metabolites	8 metabolite fit	12 metabolite fit	16 metabolite fit
Acetate (Ace)			x
Aspartate (Asp)			x
Choline (Cho)		x	x
Phosphorylcholine (PC)	x	x	x
Glycerophosphorylcholine (GPC)		x	x
Phosphorylethanolamine (PE)	x	x	x
Creatine (Cr)		x	x
Phosphocreatine (PCr)	x	x	x
N-acetyl aspartate (NAA)	x	x	x
N-acetyl aspartyl glutamate (NAAG)		x	x
Gamma-aminobutyric acid (GABA)			x
Glutamate (Glu)	x	x	x
Glutamine (Gln)	x	x	x
Glutathione (GSH)	x	x	x
Myo-inositol (mIns)	x	x	x
Taurine (Tau)			x
Macromolecules (MM)	x	x	x

Statistical analysis

To assess the reproducibility for glutamate, and the major spectral components N-acetyl aspartate, creatine and choline, a test-retest reliability test was performed (SPSS 15.0, Chicago, IL, USA) for each sequence and VOI, by calculating the intraclass correlation coefficient using a two-way mixed model ANOVA. We reported the average measures ICC, since it takes into account the average of the values of the two scan sessions. A negative ICC indicates that the measurement is not reliable.

Table 2: ICC's and p-values for measurement of glutamate concentrations, using sLASER and STEAM in a frontal and occipital VOI, for three different fitting procedures.

	sLASER				STEAM			
	frontal		occipital		frontal		occipital	
	ICC	p	ICC	p	ICC	p	ICC	p
8 metabolite fit	0.77	0.04	0.28	0.35	0.17	0.41	-3.62	0.08
12 metabolite fit	0.65	0.09	0.61	0.14	0.58	0.14	0.42	0.20
16 metabolite fit	0.80	0.03	0.34	0.31	0.56	0.15	-0.81	0.67

Table 3: Glutamate concentrations (average ± SD, in mM) at the first and second measurement, using sLASER and STEAM in a frontal and occipital VOI, for three different fitting procedures.

	sLASER				STEAM			
	frontal		occipital		frontal		occipital	
	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2
8 metabolite fit	12.4±1.8	11.6±1.7	10.5±1.7	10.8±2.5	12.3±1.5	10.5±1.9	8.0±0.9	9.2±0.9
12 metabolite fit	12.6±1.7	11.5±2.1	10.8±1.7	12.1±3.5	18.1±4.0	16.9±4.0	11.6±3.9	13.7±2.8
16 metabolite fit	13.6±2.3	12.5±2.5	11.2±1.9	11.7±2.1	19.8±3.9	19.3±5.1	12.9±6.3	15.9±2.4

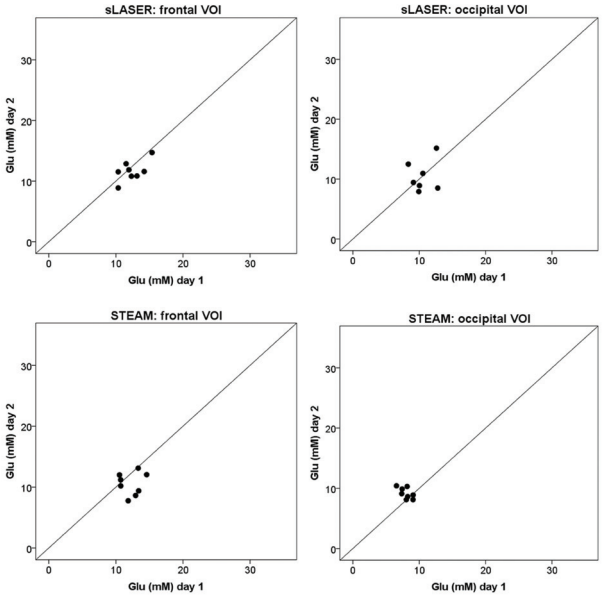

Figure 3: Glutamate concentrations calculated with an 8 metabolite fit at day 1 (x-axis) and day 2 (y-axis). The line x=y represents a correlation of +1 between the two measurements. A point-spread along the line x=y represents detection of mainly physiological variation, a point-spread perpendicular to the line x=y represents detection of mainly methodological variation

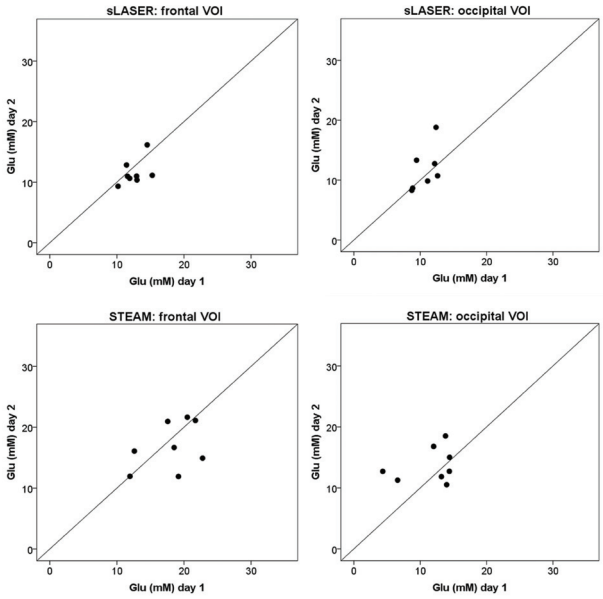

Figure 4: Glutamate concentrations calculated with a 12 metabolite fit at day 1 (x-axis) and day 2 (y-axis). The line x=y represents a correlation of +1 between the two measurements. A point-spread along the line x=y represents detection of mainly physiological variation, a point-spread perpendicular to the line x=y represents detection of mainly methodological variation

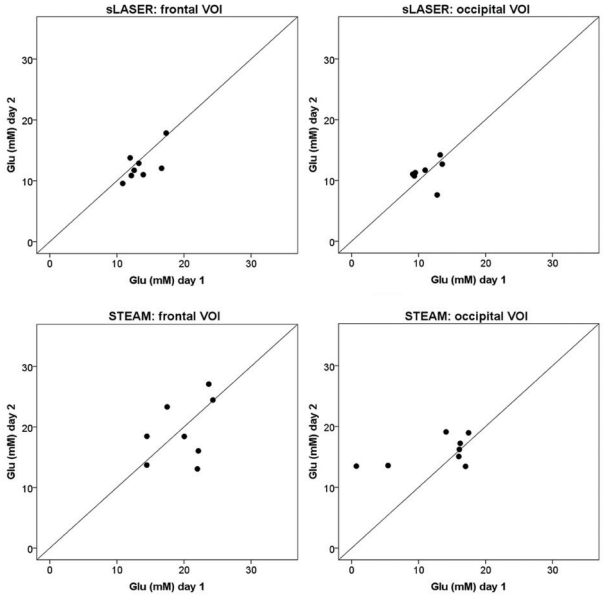

Figure 5: Glutamate concentrations calculated with a 16 metabolite fit at day 1 (x-axis) and day 2 (y-axis). The line x=y represents a correlation of +1 between the two measurements. A point-spread along the line x=y represents detection of mainly physiological variation, a point-spread perpendicular to the line x=y represents detection of mainly methodological variation.

Table 4: ICC's and p-values for measurement of N-acetyl aspartate concentrations, using sLASER and STEAM in a frontal and occipital VOI, for three different fitting procedures.

	sLASER				STEAM			
	frontal		occipital		frontal		occipital	
	ICC	p	ICC	p	ICC	p	ICC	p
8 metabolite fit	0.44	0.44	0.24	0.38	-0.45	0.68	-1.85	0.91
12 metabolite fit	0.50	0.19	0.81	0.03	0.05	0.47	-1.12	0.83
16 metabolite fit	0.54	0.17	0.58	0.16	-0.31	0.64	-1.15	0.83

Table 5: N-acetyl aspartate concentrations (average ± SD, in mM) at the first and second measurement, using sLASER and STEAM in a frontal and occipital VOI, for three different fitting procedures.

	sLASER				STEAM			
	frontal		Occipital		frontal		occipital	
	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2
8 metabolite fit	15.4±3.0	14.1±1.1	15.2±2.3	16.0±1.5	11.5±3.6	9.3±1.2	9.0±0.9	9.8±1.7
12 metabolite fit	15.5±2.4	14.0±1.1	15.6±2.1	16.2±1.9	23.8±3.5	22.1±2.0	21.1±1.7	23.8±4.7
16 metabolite fit	15.3±2.4	13.9±1.1	15.5±2.0	16.0±1.3	23.8±4.4	22.4±2.6	21.1±2.1	23.9±4.5

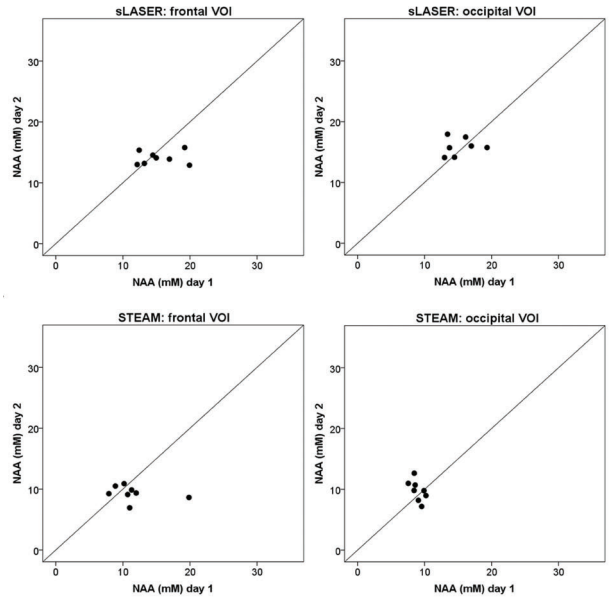

Figure 6: NAA concentrations calculated with an 8 metabolite fit at day 1 (x-axis) and day 2 (y-axis). The line x=y represents a correlation of +1 between the two measurements. A point-spread along the line x=y represents detection of mainly physiological variation, a point-spread perpendicular to the line x=y represents detection of mainly methodological variation.

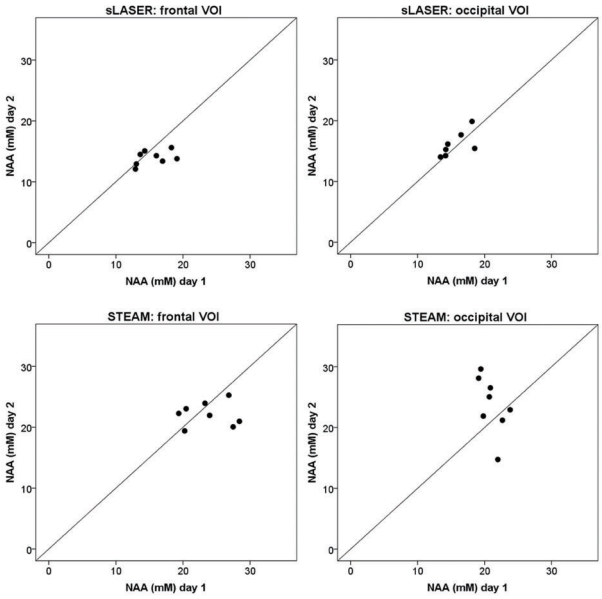

Figure 7: NAA concentrations calculated with a 12 metabolite fit at day 1 (x-axis) and day 2 (y-axis). The line x=y represents a correlation of +1 between the two measurements. A point-spread along the line x=y represents detection of mainly physiological variation, a point-spread perpendicular to the line x=y represents detection of mainly methodological variation.

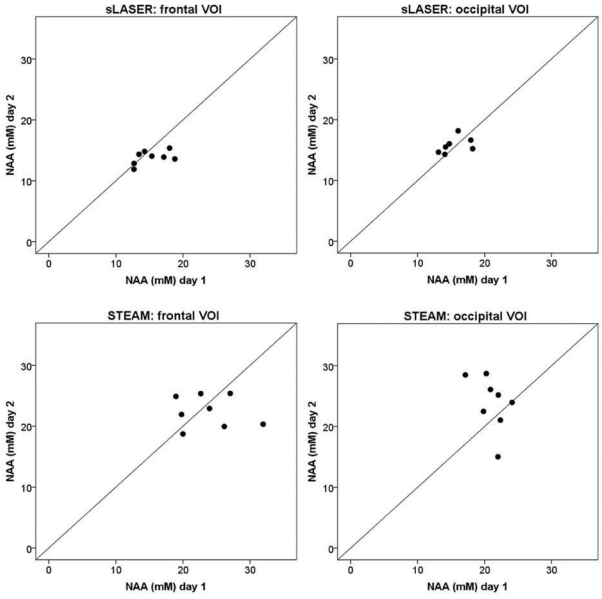

Figure 8: NAA concentrations calculated with a 16 metabolite fit at day 1 (x-axis) and day 2 (y-axis). The line x=y represents a correlation of +1 between the two measurements. A point-spread along the line x=y represents detection of mainly physiological variation, a point-spread perpendicular to the line x=y represents detection of mainly methodological
variation.

Table 6: ICC's and p-values for measurement of creatine concentrations, using sLASER and STEAM in a frontal and occipital VOI, for three different fitting procedures.

	sLASER				STEAM			
	frontal		Occipital		frontal		occipital	
	ICC	p	ICC	P	ICC	p	ICC	p
8 metabolite fit	0.16	0.41	0.15	0.42	0.08	0.46	-0.17	0.58
12 metabolite fit	0.09	0.45	0.60	0.15	-0.33	0.64	-0.03	0.52
16 metabolite fit	-0.67	0.74	0.28	0.35	-0.77	0.77	0.41	0.25

Table 7: Creatine concentrations (average ± SD, in mM) at the first and second measurement, using sLASER and STEAM in a frontal and occipital VOI, for three different fitting procedures.

	sLASER				STEAM			
	frontal		Occipital		frontal		occipital	
	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2
8 metabolite fit	11.4±1.8	10.9±0.6	9.9±1.3	9.9±0.7	8.9±2.1	7.4±0.4	7.1±0.8	6.9±1.4
12 metabolite fit	11.0±1.6	10.3±0.7	9.4±1.2	11.1±3.0	16.0±1.5	14.7±1.3	14.4±2.4	15.2±5.3
16 metabolite fit	12.1±1.7	12.0±0.9	10.1±1.2	9.9±1.1	18.6±1.8	18.2±1.8	14.4±4.5	17.8±2.7

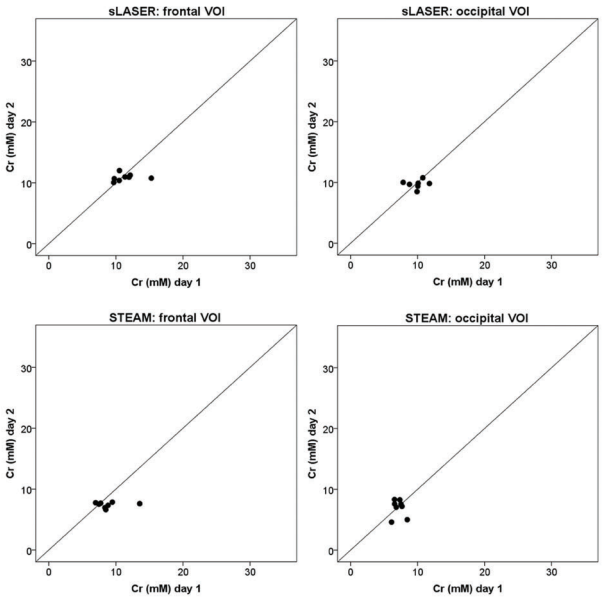

Figure 9: Creatine concentrations calculated with an 8 metabolite fit at day 1 (x-axis) and day 2 (y-axis). The line x=y represents a correlation of +1 between the two measurements. A point-spread along the line x=y represents detection of mainly physiological variation, a point-spread perpendicular to the line x=y represents detection of mainly methodological variation.

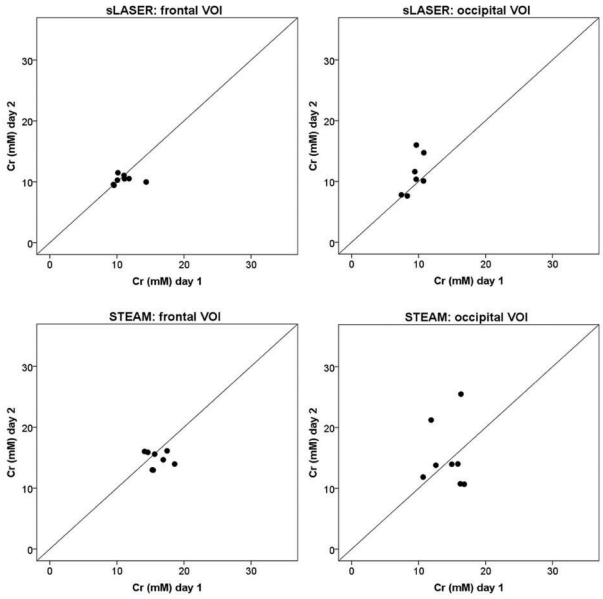

Figure 10: Creatine concentrations calculated with a 12 metabolite fit at day 1 (x-axis) and day 2 (y-axis). The line x=y represents a correlation of +1 between the two measurements. A point-spread along the line x=y represents detection of mainly physiological variation, a point-spread perpendicular to the line x=y represents detection of mainly methodological variation.

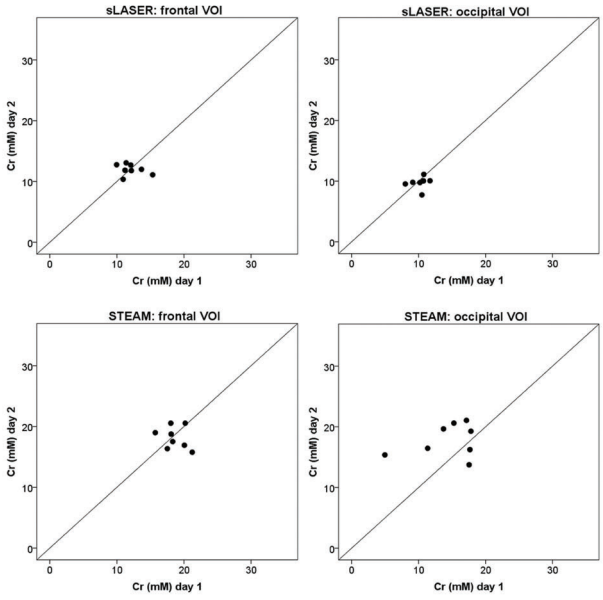

Figure 11: Creatine concentrations calculated with a 16 metabolite fit at day 1 (x-axis) and day 2 (y-axis). The line x=y represents a correlation of +1 between the two measurements. A point-spread along the line x=y represents detection of mainly physiological variation, a point-spread perpendicular to the line x=y represents detection of mainly methodological variation.

Table 8: ICC's and p-values for measurement of choline concentrations, using sLASER and STEAM in a frontal and occipital VOI, for three different fitting procedures.

	sLASER				STEAM			
	frontal		occipital		frontal		occipital	
	ICC	p	ICC	P	ICC	p	ICC	p
8 metabolite fit	0.08	0.46	0.60	0.14	0.22	0.38	0.54	0.16
12 metabolite fit	0.39	0.27	0.74	0.06	0.36	0.28	-0.01	0.50
16 metabolite fit	-0.32	0.64	0.58	0.16	-0.72	0.76	0.12	0.43

Table 9: Choline concentrations (average ± SD, in mM) at the first and second measurement, using sLASER and STEAM in a frontal and occipital VOI, for three different fitting procedures.

	sLASER				STEAM			
	frontal		Occipital		frontal		occipital	
	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2
8 metabolite fit	3.2±0.8	2.9±1.1	3.1±1.0	2.5±0.9	3.2±1.9	1.7±0.6	1.7±0.4	2.1±1.2
12 metabolite fit	2.9±0.5	2.7±0.3	2.3±0.4	2.4±0.6	5.4±1.4	4.2±0.6	2.8±0.5	3.2±0.9
16 metabolite fit	2.8±0.6	2.7±0.4	2.3±0.4	2.2±0.1	7.1±1.5	4.2±0.8	5.3±2.2	5.0±2.6

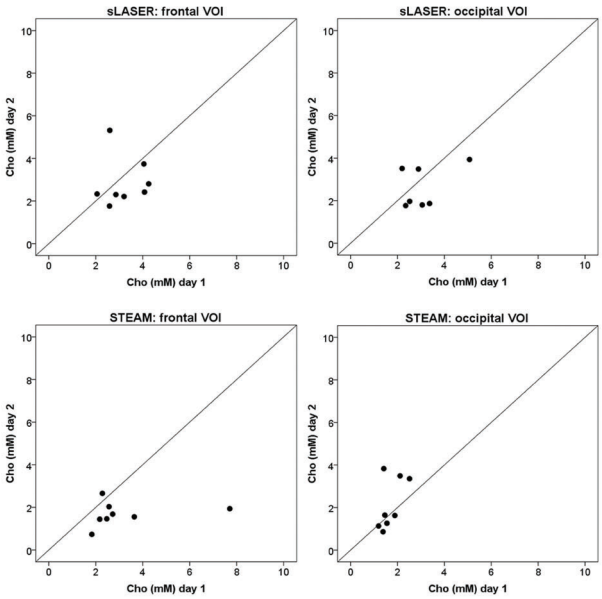

Figure 12: Choline concentrations calculated with an 8 metabolite fit at day 1 (x-axis) and day 2 (y-axis). The line x=y represents a correlation of +1 between the two measurements. A point-spread along the line x=y represents detection of mainly physiological variation, a point-spread perpendicular to the line x=y represents detection of mainly methodological variation.

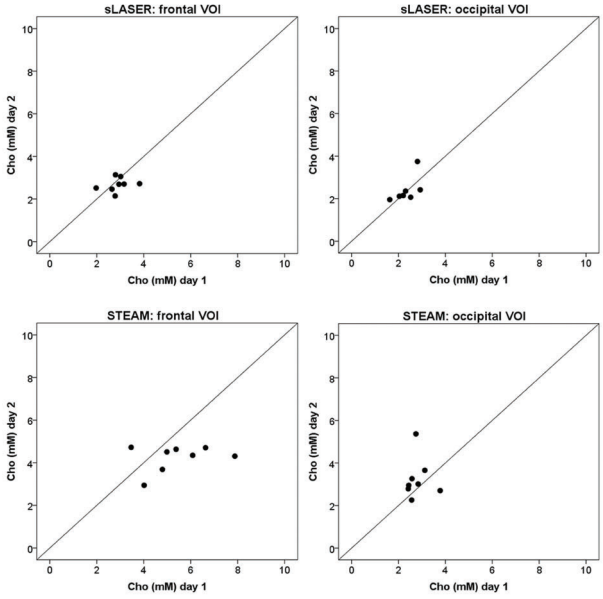

Figure 13: Choline concentrations calculated with a 12 metabolite fit at day 1 (x-axis) and day 2 (y-axis). The line x=y represents a correlation of +1 between the two measurements. A point-spread along the line x=y represents detection of mainly physiological variation, a point-spread perpendicular to the line x=y represents detection of mainly methodological variation.

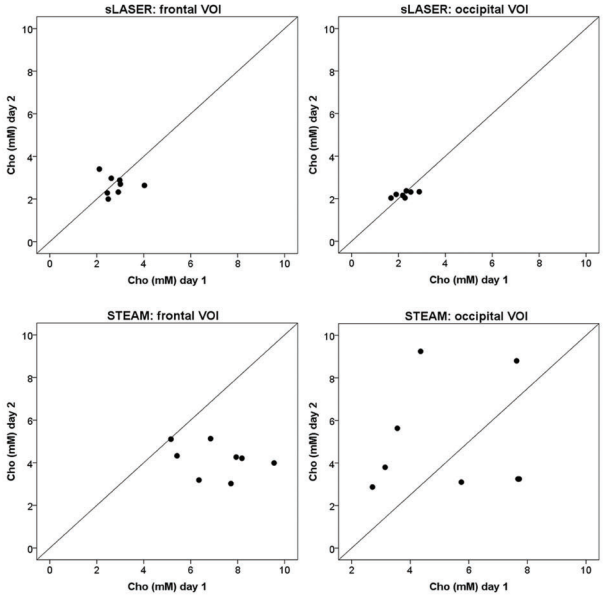

Figure 14: Choline concentrations calculated with a 16 metabolite fit at day 1 (x-axis) and day 2 (y-axis). The line x=y represents a correlation of +1 between the two measurements. A point-spread along the line x=y represents detection of mainly physiological variation, a point-spread perpendicular to the line x=y represents detection of mainly methodological variation.

Results

Glutamate concentrations for all subjects at the two scan sessions and as determined by the three different fitting procedures are shown in **Figures 3-5**. ICC's and p-values are shown in **Table 2**.

Average metabolite concentrations are shown in **Table 3**. In the frontal lobe the sLASER provides significant ICC's when fitting with 8 or 16 metabolite basis sets.

To evaluate the precision of the quantification of the most commonly obtained metabolites, average metabolite concentrations, ICC's and p-values are shown in the **Figures 6-8** and **Tables 4-5** for NAA, **Figures 9-11** and **Tables 6-7** for Cr and **Figures 12-14** and **Tables 8-9** for Cho.

For the occipital lobe a significant ICC was found only for NAA measured with sLASER and fitted with the 12 metabolite basis set.

Discussion

In this study we estimated the reproducibility of glutamate measurements by using the STEAM and sLASER sequence at 7T in two different areas of the human brain. As compared to the commonly used STEAM sequence, sLASER seems to be a more robust method for determining glutamate levels. It produces similar results at different time-points and is sensitive enough to detect physiological differences between subjects. Particularly in the frontal brain region, which plays an important role in psychiatric disorders [13-17], glutamate concentrations measured with sLASER show a high reproducibility.

Concentrations of NAA, creatine and choline are expected to remain stable over subjects, particularly in the small age range used in this study [18;19], hence one would expect lower ICC's for these metabolites, as the between subjects variance is low. The results for sLASER indeed show that the ICC's for NAA, creatine and choline are lower than the ICC computed for glutamate. This has already been shown for sLASER at 3 T [20]. In contrast to glutamate, NAA, creatine and choline do not suffer from overlap with other metabolites at lower field strength and show low between subjects variances. Therefore, measurements of these metabolites at 7T only result in a higher SNR, while the low ICC's remain.

We note that, in contrast to the sLASER measurements, a clear difference in variance can be observed between the two measurements using STEAM (see for instance figure 3). This suggests that the STEAM sequence is more sensitive than the sLASER sequence to external factors that are apparently difficult to control as we tried to keep the conditions in our experiments the same as much as possible.

As mentioned before, STEAM suffers from reduced localization accuracy as compared to sLASER, which may compromise the precision of the measurement. Also, the longer measurements times used with the STEAM sequence to partly compensate SNR loss, may have caused reduced stability. Additionally, a fitting procedure including 12 or more metabolite basis sets seems to more robustly display measured metabolite levels. It is known that omitting basis sets from metabolites that are indeed present in the tissue of interest leads to a systematic bias and potential overlap of the fitted metabolite resonances and thereby detects physiological and methodological variations less accurately [21;22]. This seems to be the case in a fitting procedure that includes basis sets for only 8 metabolites, for which we generally observed small within and between subjects variations, whereas fitting procedures including basis sets for 12 or 16 metabolites show larger variations within and between subjects.

Several limitations have to be considered when interpreting the results of this study. First, only eight subjects were examined. Although this was not enough to establish the reliability for the STEAM sequence, it was enough to establish the reliability of the sLASER sequence. This finding speaks in favour of the sLASER sequence. On the other hand, application of the STEAM sequence in clinical studies is easier since it does not require additional hardware modifications. To reach a short echo time on the system we used, the sLASER required a dual transmit option. This may not be routinely available on all 7T MR systems and it also requires slightly more scan preparation, e.g. determination of the optimal phase of the two input channels. If fully automated however, this would only require several seconds.

A potential cause of variation between repeated measurements might be the manual positioning of the VOIs. Differences in location may result in different contributions of gray matter (GM), white matter (WM) and CSF, which may affect the measured metabolite levels [20]. However, the ICC's of gray and white matter content between the first and second measurement in the frontal (GM: ICC=0.89, $p<0.01$; WM: ICC=0.87, $p<0.01$) and the occipital voxel (GM: ICC=0.69, $p=0.07$; WM: ICC=0.67, $p=0.09$) indicate only small variations in positioning. Larger variations in positioning and thus slightly smaller ICC's for gray and white matter content, could explain the fact that no significant ICC's for metabolite levels were found in the occipital region. Also, we corrected the measured metabolite levels for contribution of GM, WM and CSF by tissue segmentation of the VOIs based on the T_1 weighted image. While this corrects for the differences in water content and assumed absence of glutamate in CSF, it does not correct for differences in glutamate between gray and white matter. However, it is not plausible that systematic variations in gray and white matter content of the VOIs are causing high

ICC's for glutamate concentrations, since we did not find significant correlations between glutamate concentrations and gray matter content, and including gray or white matter fractions as a regressor in the analyses did not yield different results.

We conclude that the sLASER sequence can be successfully applied for glutamate measurements in the human brain at 7T. MR spectra acquired at different time-points, and in a well-controlled population, are comparable and robust and the variance is mainly caused by physiological differences between subjects, since the methodological variation is reduced when using sLASER compared to STEAM. This is particularly beneficial when studying populations that are difficult to recruit, since sLASER requires a smaller sample size than STEAM to detect physiological differences between subjects.

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6

GABA and glutamate in schizophrenia: a 7T ^{1}H-MRS study

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Abstract

Schizophrenia is characterized by a loss of brain tissue, which may represent an ongoing pathophysiological process. A possible mechanism that may be involved is the glutamatergic system. Particularly hypofunction of the NMDA-type of glutamate receptor may be involved. NMDA-receptor hypofunction leads to insufficient excitatory activity, disrupting the function of GABAergic inhibitory neurons, which in turn may downregulate their activity. Indeed, postmortem and animal studies show altered expression of markers of GABAergic neurotransmission. In this study, GABA/creatine ratios, and glutamate, NAA, creatine and choline concentrations in the prefrontal and occipital cortex were measured in 17 patients with schizophrenia and 23 healthy controls using proton magnetic resonance spectroscopy at an ultra-high magnetic field strength of 7T. Significantly lower prefrontal GABA/Cr ratios were found in patients with schizophrenia as compared to healthy controls, which was strongly associated with level of cognitive functioning. No significant change in the GABA/Cr ratio was found between patients and controls in the occipital cortex. No significant changes in levels of glutamate, NAA, creatine, and choline between patients and controls were found in the prefrontal and occipital cortex. These results suggest a (compensatory) role for GABA through altered inhibitory neurotransmission in the prefrontal cortex may be present in schizophrenia.

Introduction

Schizophrenia is characterized by a loss of brain tissue, that is at least in part progressive, and which may represent an ongoing pathophysiological process [1]. This loss of brain tissue may be explained by reduced neuropil rather than neuronal loss, suggesting abnormal synaptic plasticity and cortical microcircuitry [2]. The mechanisms underlying brain tissue loss in schizophrenia are not known. A possible mechanism that may be involved is the glutamatergic system. Glutamate (Glu) is the most important excitatory neurotransmitter in the central nervous system (CNS). Indeed, glutamate levels are reduced in the frontal brain region in schizophrenia, moreover, glutamate levels appear to decrease progressively with age [3]. Particularly hypofunction of the NMDA-type of glutamate receptor may be involved [4-6]. NMDA-receptor hypofunction leads to insufficient excitatory activity, disrupting the function of GABAergic inhibitory neurons, which in turn may downregulate their activity [7;8]. GABA (gamma-aminobutyric acid) is the major inhibitory neurotransmitter in the CNS. GABA has also been implicated in schizophrenia based on postmortem and animal studies.

Indeed, several postmortem and animal studies showed reduced mRNA expression of presynaptic markers of GABAergic neurotransmission in subpopulations of GABAergic interneurons in the prefrontal cortex in (models for) schizophrenia [9-11]. Levels of mRNA encoding for the 67 kDa isoform of glutamic acid decarboxylase (GAD67), the enzyme that facilitates decarboxylation of glutamate to synthesize GABA, were reduced in schizophrenia [12-15]. More specifically, it appears that the density of neurons with detectable levels of GAD67 is decreased but the level of GAD67 mRNA expression per neuron remains unaltered [16]. In particular, chandelier afferents innervating pyramidal cells seem to be affected [17]. Also, the expression of the GABA transporter (GAT-1) is decreased below detectable levels in chandelier axons [18;19]. Thus on the one hand, decreased GAD67 expression might attenuate GABAergic neurotransmission, while on the other hand decreased GAT-1 expression might enhance GABAergic neurotransmission [11]. In addition, the expression of subunits of the postsynaptic $GABA_A$ receptor, the receptor that produces most of the physiological actions of GABA [20], is altered. Increased expression of $GABA_A$ $\alpha 2$ receptor subunits may be a consequence of diminished synaptic GABA levels due to decreased synthesis. However, decreased expression of the $\alpha 1$, $\alpha 5$, γ and δ subunits might reflect a primary disease process since it is unlikely that this downregulation serves to compensate decreased synaptic GABA levels [21]. Overall, evidence points to a net hypofunction of subpopulations of GABAergic interneurons in the prefrontal cortex

in schizophrenia [11]. However, postmortem studies are usually based on patients that have been chronically ill for many years. To establish possible influences of glutamate and GABA levels in early stages of the disease, *in vivo* measurements using magnetic resonance spectroscopy (MRS) are the method of choice.

Until now, a total of six studies investigated GABA levels *in vivo* in the brains of patients with schizophrenia, using proton MRS (^{1}H -MRS) [22-27]. One study examined early stage schizophrenia patients only and showed reduced GABA levels in the left basal ganglia (BG), but not in the frontal and parieto-occipital (POC) lobes [22]. Three studies examined chronically ill patients only [23;24]. One of these studies showed elevated GABA levels in the anterior cingulate cortex (ACC) and POC [23]. The other two studies showed unaltered GABA levels in the medial frontal brain region [24;27] and left basal ganglia, with higher left BG GABA levels in patients taking typical antipsychotics versus patients taking atypical antipsychotics [24]. Two studies examined patients with early stage schizophrenia as well as patients with chronic schizophrenia [25;26], showing reduced GABA levels in the visual cortex [25], and unaltered GABA levels in the dorsolateral prefrontal cortex (DLPFC) in both patient-groups, as well as elevated GABA levels in the medial prefrontal cortex (MPFC) in patients that were antipsychotic medication free at the time of the scan but not in medicated patients, [26]. Thus, overall, increases and decreases as well as normal GABA levels have been reported in schizophrenia that may or may not depend on disease stage, medication intake and brain area. However, while these studies have been done at conventional magnetic field strengths (such as 3T), this may have left subtle changes in these metabolite levels unrevealed.

Performing ^{1}H-MRS at ultra-high magnetic field strength (7T) has two clear advantages. The sensitivity of the measurements of metabolite concentrations is increased because of the increased signal-to-noise ratio (SNR) and the increased spectral resolution allows for a better separation of the individual metabolite spectra. For instance, at a magnetic field strength of 7T it is now possible to adequately separate the glutamate and glutamine signals resulting in a higher accuracy of glutamate measurement [28]. However, detection of metabolites at a higher magnetic field strength is complicated by reduced accuracy in localization of the region of interest [29;30]. The sLASER (semi-localized by adiabatic selective refocusing) sequence enables ^{1}H-MRS of the human brain at 7T with increased localization accuracy, SNR and reliability [29;31]. Despite the increased SNR the measurement of GABA is not straightforward because the concentration of GABA is low compared to other brain metabolites and the GABA signal is obscured by other overlapping metabolites with higher signal intensity. To overcome this problem, spectral editing techniques can be applied to isolate the GABA signal [32]. In this

study we used the sLASER sequence combined with editing techniques (MEGA-sLASER) which allows for accurate and time-efficient detection of GABA [30].

The purpose of this study was to measure GABA and glutamate levels in patients with schizophrenia and healthy control subjects. We collected data from the medial prefrontal region, which is known to show structural abnormalities in schizophrenia, and the medial occipital region as a control area. Based on prior studies, we hypothesized that frontal GABA and glutamate levels are reduced and progressively decreasing with age, and occipital GABA and glutamate levels remain unchanged in schizophrenia. To our knowledge, this is the first study in schizophrenia patients using ^{1}H-MRS at a magnetic field strength of 7T.

Methods

Subjects

A total of 41 individuals participated in the study, including 18 patients with schizophrenia, and 23 healthy control participants matched to the patients for age, sex and their parent's socio-economic status. Healthy participants had no major psychiatric or neurological history, no history of drug or alcohol abuse, and no first-degree relatives with psychiatric or neurological disorders. The study was approved by the Medical Ethics Committee of the University Medical Center Utrecht, The Netherlands, and performed according to the directives of the Declaration of Helsinki (amendment of Seoul, 2008). All participants provided written informed consent prior to the examination.

All individuals underwent psychiatric assessment using the Comprehensive Assessment of Symptoms and History [33]. Symptom severity was assessed using the Positive and Negative Syndrome Scale (PANSS) [34]. Current daily dosage and cumulative life-time dosage of antipsychotic medication in haloperidol equivalents were calculated [35]. In addition, current or prior use of benzodiazepines was established. One patient did not undergo MR scanning because of a relapse in symptoms, thus the total number of patients that we report on is 17. All individuals underwent a general cognitive assessment using the full Wechsler Adult Intelligence Scale (WAIS) - III [36], which revealed a total intelligence quotient (TIQ) as well as a verbal and performance IQ, and working memory, perceptual reasoning, and verbal comprehension indices. Cognitive assessment could be successfully completed in all but three subjects. The WAIS-III was not measured in 3 healthy subjects because of logistical reasons.

No differences between the groups were found for age, parental completed level of education and sex. Groups differed significantly in completed level of education, with patients having lower education, and on general cognitive functioning, with patients having a lower level of intelligence, as compared to the controls (**Table 1**).

Table 1: Demographic characteristics

Characteristic	Patients	Controls	Statistic (p-value based on t, χ2)
Sex, M/F, N	13/4	16/7	0.45
Age, mean (SD), min-max, years	27.6 (6.1), 20.6-41.7	27.7 (5.3), 21.5-40.9	0.97
Education level, mean (SD)			
Participants			0.08
Parents			0.61
Total IQ, mean (SD)	92.8 (14.5)	108.3 (13.0)	0.002
Duration of illness, mean (SD), min-max, months	77.4 (82.1), 1-213		
PANSS, mean (SD)			
Total	53.1 (12.7)		
Negative	12.9 (4.5)		
Positive	12.7 (5.1)		
General	27.5 (7.3)		
Antipsychotic medication Current daily dosage in haloperidol equivalents, N, mean (SD)			
Clozapine	7, 4.26 (2.15)		
Atypical	14, 5.48 (2.92)		
Olanzapine	8, 6.84 (2.79)		
Risperidone	1, 5.56		
Aripiprazole	4, 4.04 (1.41)		
Sulpiride	1, 0.29		
Antipsychotic medication Cumulative dosage in haloperidol equivalents, N, mean (SD)			
Typical	5, 572 (483)		
Clozapine	8, 6031 (6846)		
Atypical	16, 3903 (4003)		
Olanzapine	15, 2647 (3904)		
Risperidone	5, 1934 (3219)		
Aripiprazole	7, 1590 (1418)		
Quetiapine	4, 445 (638)		
Benzodiazepines current N (%), cumulative N (%)	6 (35%), 11 (65%)		

MR acquisition

All investigations were performed on a 7 T whole body MR scanner (Philips, Cleveland, OH, USA). A birdcage transmit head coil was used in dual transmit driven by 2x4 kW amplifiers, in combination with a 32-channel receive coil (both Nova Medical, Inc., Burlington, MA, USA).

For anatomical reference and gray and white matter tissue classification a T_1-weighted magnetization prepared rapid gradient echo (MP-RAGE) sequence was obtained (450 slices, slice thickness = 0.8 mm, TR = 7 ms, TE = 3 ms, flip angle = 8 degrees, FOV = 250 x 200 x 180 mm, 312 x 312 acquisition matrix, SENSE factor 2.7, scan duration = 408 s).

For the assessment of glutamate and the major spectral components NAA, creatine and choline, ^{1}H-MRS experiments were conducted using a sLASER sequence (semi-localized by adiabatic selective refocusing; TE = 28 ms, 32 averages, TR = 5 s) (**Figure 1A**). Voxels (2 x 2 x 2 cm^3) were located in the medial prefrontal and medial occipital lobe (**Figure 2**). Non-water-suppressed spectra were obtained for quantification (carrier frequency was set to the chemical shift of H_2O, acquisition time = 10 s).

GABA-edited ^{1}H-MRS experiments were conducted using a MEGA-sLASER sequence (TE = 74 ms, 64 averages, TR = 4 s) (**Figure 1B**). Voxels (2.5 x 2.5 x 2.5 cm^3) were located in the medial frontal and medial occipital region (Figure 2). Prior to the MRS exams, second order B_0 shimming was applied using the FASTERMAP algorithm at the voxel of interest [37;38]. Second, at this location, a high B_1 field was generated to minimize chemical shift displacement artefacts [39]. The highest possible B_1 field was generated by optimizing the phase of both transmit channels to locally assure constructive B_1 interferences [29].

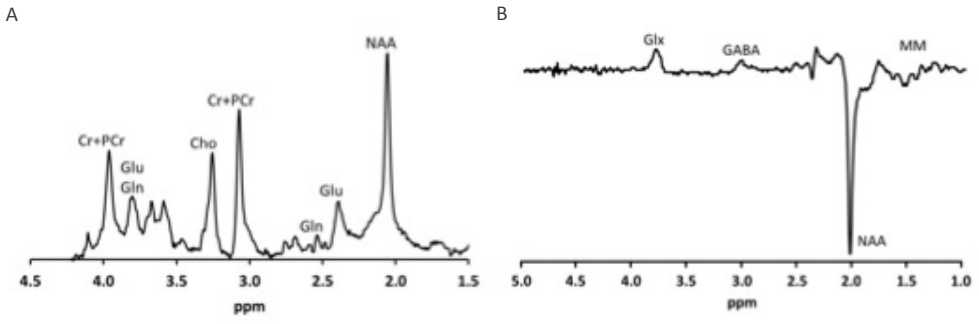

Figure 1: Typical metabolite spectra (A) as recorded using the sLASER sequence and (B) as recorded using the MEGA-sLASER sequence.z

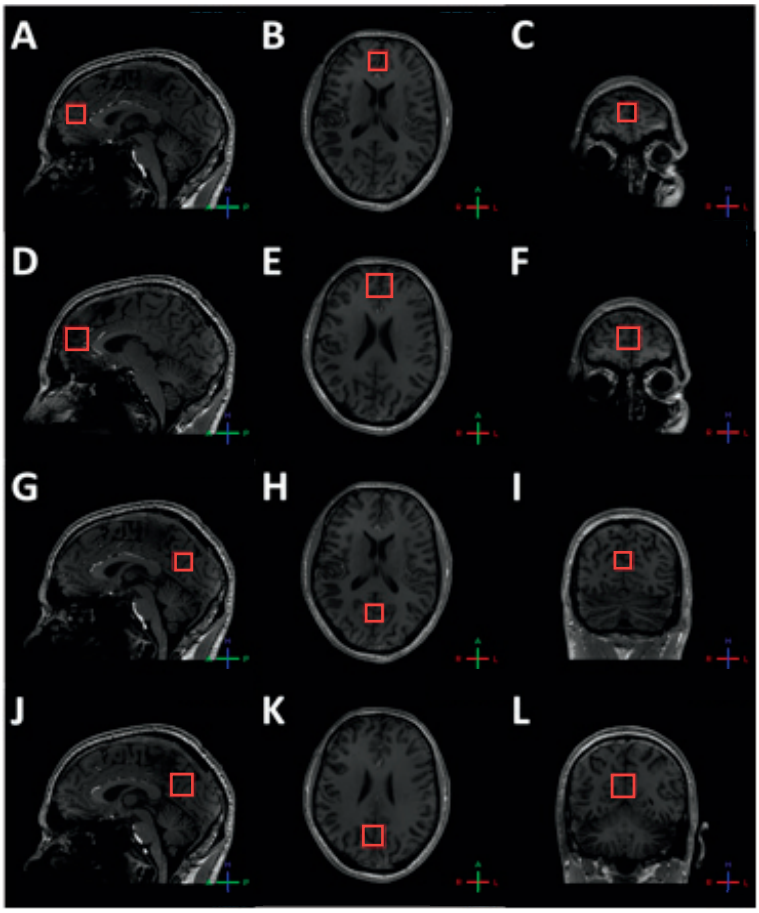

Figure 2: Voxel placement. A: frontal sLASER voxel, sagittal view; B: frontal sLASER voxel, axial view; C: frontal sLASER voxel, coronal view; D: frontal MEGA-sLASER voxel, sagittal view; E: frontal MEGA-sLASER voxel, axial view; F: frontal MEGA-sLASER voxel, coronal view; G: occipital sLASER voxel, sagittal view; H: occipital sLASER voxel, axial view; I: occipital sLASER voxel, coronal view; J: occipital MEGA-sLASER voxel, sagittal view; K: occipital MEGA-sLASER voxel, axial view; L: occipital MEGA-sLASER voxel, coronal view.

Spectral fitting and quantification

Retrospective phase and frequency alignment was performed on all data sets of each measurement [40]. Fitting of the sLASER spectra was performed with LCModel-based software implemented in Matlab [41], which uses a priori knowledge of the spectral components to fit metabolite resonances [42]. The following 16 metabolites and a measured macromolecular baseline [43] were fitted to the spectra: acetate, aspartate, choline (Cho), phosphorylcholine (PC), glycerophosphorylcholine (GPC), phosphorylethanolamine (PE), creatine (Cr), phosphocreatine (PCr), N-acetyl

aspartate (NAA), N-acetyl aspartyl glutamate (NAAG), GABA, Glu, glutamine (Gln), glutathione (GSH), myo-inositol (mIns), and taurine (Tau). Levels of Glu, total NAA (NAA+NAAG), total creatine (Cr+PCr) and total choline (Cho+PC+GPC+PE) were estimated using the water signal as an internal reference and calculated as follows:

$$[\mathrm{met}] = \left(\frac{\frac{\mathrm{signal_{met}}}{\mathrm{signal_{water}}} * \left(\mathrm{volGM} * [\mathrm{water_{GM}}] + \mathrm{volWM} * [\mathrm{water_{WM}}] + \mathrm{volCSF} * [\mathrm{water_{pure}}] \right)}{\mathrm{volGM} + \mathrm{volWM}} \right)$$

Where [met] is the metabolite concentration, $\mathrm{signal_{met}}$ is the fitted signal intensity of the metabolite, accounting for the number of protons, and $\mathrm{signal_{water}}$ is the fitted signal intensity of water, accounting for the number of protons; volGM, volWM and volCSF are respectively the gray matter fraction, white matter fraction and cerebrospinal fluid (CSF) fraction in the voxel; and [$\mathrm{water_{GM}}$], [$\mathrm{water_{WM}}$], and [$\mathrm{water_{pure}}$] are respectively the water concentration in gray matter, white matter or CSF. For determining the contribution of gray matter, white matter and CSF of each voxel, the software package SPM8 was used to segment the T_1-weighted image. In the T_1-weighted image, the position of the ^{1}H-MRS voxel was determined, after which the amount of gray matter, white matter and CSF in the ^{1}H-MRS voxel was computed. To account for differences in transverse relaxation between water and metabolites, a correction was applied based on reported T_2 values at 7T of 47 ms on average for water and 107 ms assumed for the metabolites [44]. Statistical analysis of the gray and white matter fractions in the frontal and occipital MEGA-sLASER (GABA/Cr) and sLASER (glutamate, NAA, creatine, choline) revealed correlations > 0.95 for both gray and white matter fractions in the two voxels.

Fitting of the MEGA-sLASER spectra was performed by frequency-domain fitting of the GABA and creatine resonances to a Lorentzian line-shape function in Matlab. GABA levels were expressed as the ratios of their peak areas relative to the peak areas of the creatine resonance.

Because of poor spectral quality as established by a Cramér-Rao lower bound (CRLB) of more than 20% and visual inspection, some data were excluded from the study. Frontal MRS results are based on 18 healthy subjects and 14 patients and occipital MRS results are based on 17 healthy subjects and 15 patients. Frontal GABA-edited MRS results are based on 19 healthy subjects and 13 patients and occipital GABA-edited MRS results are based on 19 healthy subjects and 15 patients.

Because of poor quality of the T_1-weighted MP-RAGE sequence, in 2 healthy subjects the gray and white matter separation could not be estimated reliably in

the frontal cortex, and in 2 healthy subjects the occipital cortex. For analyses with correction for gray and white matter fractions these subjects were not included in the analyses.

Statistical analysis

Statistical analyses were performed using SPSS 21.0 (2012, Chicago, IL). Demographic characteristics were addressed for differences between the groups using Student's t-tests and χ^2-tests. Data were checked for normality of their distributions. No transformations were required on any of the data. To evaluate differences in metabolite levels between patients and controls, for each metabolite separately, multiple univariate analyses of variance were done with metabolite level as dependent variable and with group (schizophrenia patients, healthy controls), age, sex and gray and white matter fractions in the voxel as independent variables. To address whether differences between groups varied with increasing age, age-by-group interaction was added to the model when the main effect of group or age became significant. To determine whether the effect of group may be different in male and female patients, a sex-by-group interaction was added to the model when the main effect of group or sex became significant. To address whether differences between groups varied with general cognitive functioning, intelligence and intelligence-by-group interaction were added to the model when the main effect of group became significant.

In patients, possible dependencies of metabolite concentrations depended on clinical symptomatology, or on current and cumulative antipsychotic medication intake were measured through post-hoc analyses. For this purpose metabolite concentrations were correlated with PANSS positive symptom scores, PANSS negative symptom score, and PANSS total scores, as well as with current daily dosage and cumulative antipsychotic medication intake in haloperidol equivalents separated out for typical, atypical and clozapine. In case of a significant finding with atypical antipsychotic medication intake, subsequent correlations with the specific compounds were done, including olanzapine, risperidone, aripripazole, and quetiapine; also findings were assessed within the patients for benzodiazepine intake.

Results

GABA

There was a significant main effect of group on GABA/Cr ratio In the medial prefrontal cortex ($F(1,24)=7.33$, $p=0.012$), due to the patients having lower GABA/Cr ratios as compared to the healthy controls (Table 2; **Figure 3A**). This effect remained significant when corrections for gray and white matter fractions were left out of the analyses (thus adding GABA/Cr ratio data from an additional two healthy subjects) with $F(1,28)=4.91$, $p=0.035$). Please note that 3 healthy subjects had relatively high values (>0.18; see Figure 3A). When these 3 subjects are left out from the analyses, GABA/Cr levels in the prefrontal cortex are not significantly different between the groups. However, there is no reason to leave these data out of the analysis, since these data do not represent extreme values or outliers in the data and are unlikely to be due to difficulties with the analysis of the individual spectra There were no significant influences of age, and age-by-group interaction on GABA/Cr ratio in the prefrontal cortex.

In the occipital cortex there was no significant main effect of group on GABA/Cr ratios ($F(1,24)=2.35$, p=n.s.) (**Figure 3B**). There was a significant main effect of age on GABA/Cr ratio in the occipital cortex ($F(1,24)=7.88$, $p=0.01$), with older individuals having lower values, irrespective of disease. There was no significant age-by-group interaction on GABA/Cr ratio in the occipital cortex.

Glutamate

There was no significant main effect of group on glutamate level in the medial prefrontal cortex ($F(1,26)=0.97$, p=n.s.) (**Table 2; Figure 3D**). There was a significant main effect of age on glutamate levels in the prefrontal cortex, with both older patients and controls having lower levels than younger patients and controls ($F(1,26)=11.16$, $p=0.003$). Adding group-by-age interaction to the model did not significantly alter the findings. There was a significant main effect of sex ($F(1,26)=4.64$, $p=0.04$) on glutamate levels in the prefrontal cortex, with higher glutamate levels in females as compared to males, irrespective of disease. Adding group-by-sex interaction to the model did not significantly alter the findings.

In the occipital cortex there were no significant effects for group, age, and sex on glutamate level (**Figure 3E**).

Table 2: Metabolite levels (mean (SD))*

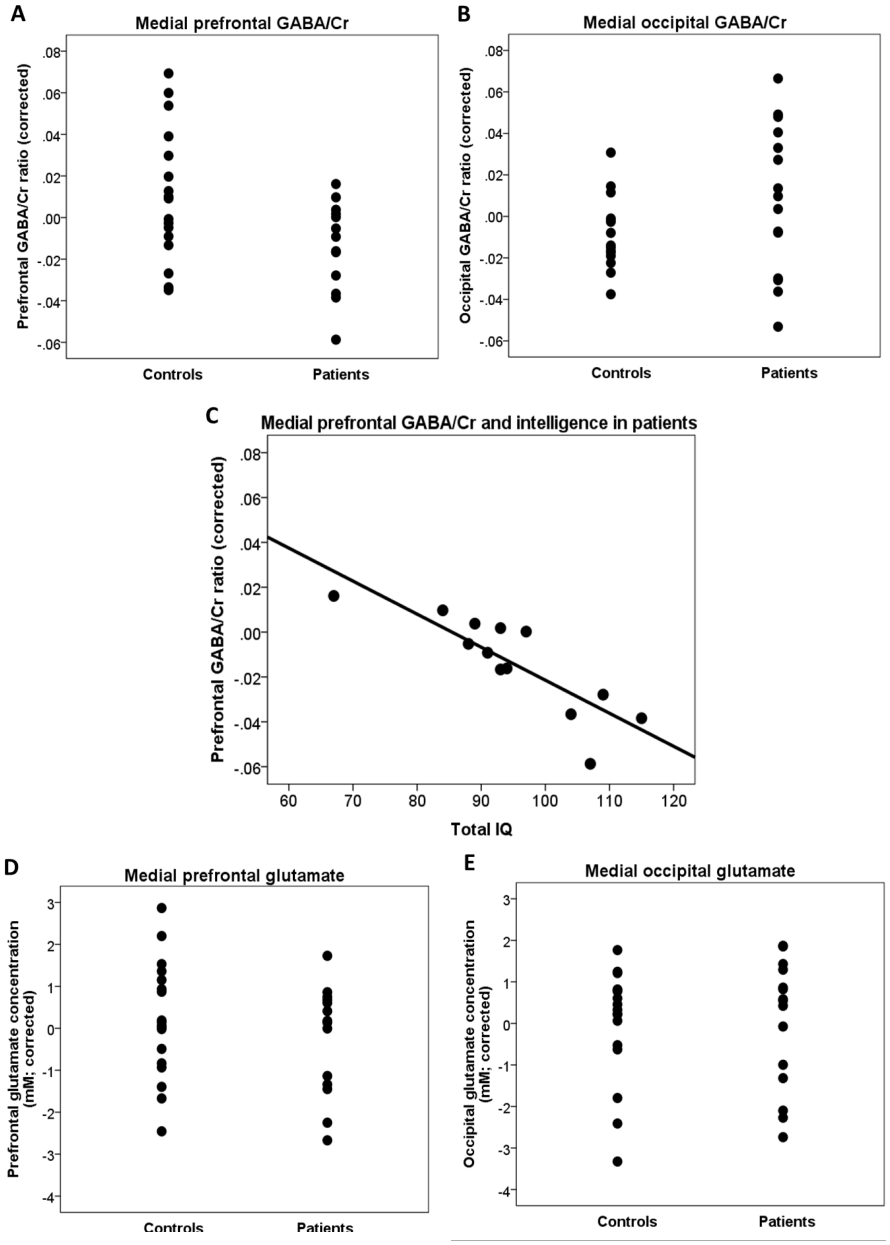

Figure 3: GABA/Cr ratios and glutamate concentrations in healthy controls and patients with schizophrenia. (A) Medial prefrontal GABA/Cr ratios. Patients show significantly lower GABA/Cr ratios as compared to healthy controls ($F(1,24)=7.33$, $p=0.012$), when correcting for age, sex, (and gray and white matter fractions); (B) Medial occipital GABA/Cr ratios. There is no significant difference between patients and controls ($F(1,24)=2.35$, p=n.s.); (C) Medial prefrontal GABA/Cr ratios in patients with schizophrenia significantly decrease with increasing total IQ ($r=-0.91$, $p=0.001$). (D) Medial prefrontal glutamate concentrations. There is no significant difference between patients and controls ($F(1,26)=0.97$, p=n.s.); (E) Medial occipital glutamate concentrations. There is no significant difference between patients and controls ($F(1,26)=0.002$, p=n.s.).

	Medial prefrontal cortex			Medial occipital cortex		
Metabolite	Patients	Controls	Statistic (F, p)	Patients	Controls	Statistic (F, p)
GABA/Cr ratio	0.12 (0.02)	0.14 (0.03)	7.33, 0.012	0.15 (0.04)	0.14 (0.03)	2.59, n.s.
Glutamate (mM)	8.48 (1.34)	8.65 (1.14)	0.97, n.s.	8.45 (1.07)	8.48 (1.26)	0.00, n.s.
NAA (mM)	9.36 (1.23)	9.85 (1.39)	1.04, n.s.	10.35 (1.26)	10.85 (1.13)	2.67, n.s.
Creatine (mM)	8.35 (1.45)	8.26 (1.43)	0.08, n.s.	8.48 (1.24)	8.28 (1.25)	0.13, n.s.
Choline (mM)	5.02 (1.96)	5.14 (1.51)	0.49, n.s.	3.48 (1.12)	3.35 (1.23)	0.00, n.s.

* Data are based on two separate ^{1}H-MRS measurements each done in two brain areas (prefrontal and occipital). A MEGA-sLASER sequence and anatomy scan were performed for assessment of GABA levels and successfully completed in 13 patients and 17 controls in the medial prefrontal cortex and in 15 patients and 15 controls in the medial occipital cortex. A sLASER sequence and anatomy scan were performed for assessment of glutamate, NAA, creatine, and choline levels and successfully completed in 14 patients and 18 controls in the medial prefrontal cortex and in 15 patients and 17 controls in the medial occipital cortex.

Other metabolites

There was a significant main effect of age ($F(1,26)=7.22$, $p=0.01$) on NAA level in the prefrontal cortex, with older patients and controls having lower levels than younger patients and controls. Adding age-by-group interaction to the model did not significantly alter the findings. There was no significant effect of group ($F(1,26)=1.04$, p=n.s.), and of sex ($F(1,26)=1.99$, p=n.s.) on NAA level in the prefrontal cortex. There was no significant main effect of group on NAA level in the occipital cortex ($F(1,26)=2.67$, p=n.s.). There was a significant main effect of sex on NAA level in the occipital cortex ($F(1,26)=6.18$, $p=0.02$), with females having higher levels than males, irrespective of disease. Adding group-by-sex interaction to the model did not alter the findings. There was no significant main effect of age and group on NAA level in the occipital cortex.

There was no significant main effect of group ($F(1,26)=0.08$, p=n.s.) on prefrontal creatine level. There was a significant main effect of age ($F(1,26)=5.21$, $p=0.03$) on prefrontal creatine level, with older patients and controls having lower levels than younger patients and controls. Adding age-by-group interaction to the model did not alter the findings. There was no significant effect of sex ($F(1,26)=0.97$, p=n.s.) on prefrontal creatine level. There was no significant main effect of group ($F(1,26)=0.13$, p=n.s.), age ($F(1,26)=2.32$, p=n.s.), and sex ($F(1,26)=1.28$, p=n.s.) on occipital creatine level. Adding age-by-group interaction to the model did not alter the findings. There were no significant effects of group ($F(1,26)=0.49$, p=n.s.), age ($F(1,26)=0.03$, p=n.s.), and sex ($F(1,26)=0.21$, p=n.s.) on prefrontal choline level. There were no significant effects of group ($F(1,26)=0.0$, p=n.s.), age ($F(1,26)=0.04$, p=n.s.), and sex ($F(1,26)=0.22$, p=n.s.) on occipital choline level.

Associations with intelligence and clinical symptoms

There was a significant interaction effect of intelligence-by-group on GABA/Cr ratio in the prefrontal cortex ($F(1,20)=4.77$, $p=0.04$), when intelligence and intelligence-by-group interaction were added to the model, that was also including group, age, sex, and gray and white matter fractions. This effect remained significant when corrections for gray and white matter fractions were left out of the analyses (thus adding GABA/Cr ratio data from an additional one healthy subject) with $F(1,23)=4.79$, $p=0.039$). This interaction effect was due to patients with a higher intelligence level having a lower GABA/Cr ratio ($r=-0.92$, $p<0.001$) (**Figure 3C**), whereas in healthy individuals no significant association between intelligence level and GABA/Cr was found. In patients, the correlation between higher level of intelligence and lower GABA/Cr ratio was found for all aspects of intelligence but revealed to be most prominently reflected in performance IQ ($r=-0.89$, $p<0.001$), followed by working memory index ($r=-0.76$, $p=0.02$), verbal IQ ($r=-0.74$, $p=0.02$), perceptual reasoning index ($r=-0.72$, $p=0.03$), and verbal comprehension index ($r=-0.70$, $p=0.04$) (all correlations corrected for gray and white matter fraction, age and sex). Indeed, when correcting for age, sex, gray and white matter fractions as well as for intelligence, the main effect of group on GABA/Cr ratio in the prefrontal cortex became highly significant with $F(1,21)=9.43$, $p=0.006$). Moreover, while occipital choline levels did not differ significantly between the patients and the controls, within the patients there was a strong positive correlation for occipital choline level with total intelligence ($r=0.85$, $p=0.001$), verbal IQ ($r=0.74$, $p=0.009$), performance IQ ($r=0.79$, $p=0.004$), verbal comprehension index ($r=0.66$, $p=0.026$), perceptual reasoning index ($r=0.70$, $p=0.017$), and working memory index ($r=0.81$, $p=0.002$).

Occipital glutamate ($r=-0.85$, $p=0.003$), occipital NAA ($r=-0.84$, $p=0.005$), and occipital choline ($r=-0.79$, $p=0.012$) levels were negatively correlated with the severity of negative symptoms. There were no other significant associations between metabolite levels in the prefrontal and occipital cortex with severity of symptomatology in patients.

Associations with antipsychotic medication and benzodiazepine intake

There was a significant negative correlation between GABA/Cr ratios in the prefrontal cortex and current daily dosage in haloperidol equivalents of atypical antipsychotic medication ($r(7)=-0.78$, $p=0.01$) and this was due to higher GABA/Cr ratio levels in the prefrontal cortex being correlated with lower level of daily

dosage of olanzapine intake (r(7)=-0.88, p=0.002). There was a significant negative correlation between glutamate level in the prefrontal cortex and cumulative dosage in haloperidol equivalents of clozapine (r(8)=-0.93, p<0.0001).

There was a significant positive correlation between current daily dosage in haloperidol equivalents of Olanzapine with level of glutamate (r=0.85, p=0.001), NAA (r=1.00, p=<0.0001), and choline (r=0.93, p=<0.0001) in the occipital cortex.

There were no other significant associations between metabolite levels in the prefrontal and occipital cortex with current or cumulative dosage of classical or atypical antipsychotic medication or clozapine.

There were no significant differences in GABA/Cr ratios in the prefrontal and occipital cortices between patients who had used benzodiazepines prior to the scan, or were using benzodiazepines at the time of the scan, although levels were on average higher in patients using benzodiazepines at the time of the scan as compared to patients who did not. In the prefrontal cortex the mean GABA/Cr ratio in patients using benzodiazepines at the time of the scan was 0.13 (SD = 0.01) as compared to 0.11 (SD = 0.02) in patients not using benzodiazepines at the time of the scan. In the occipital cortex the mean GABA/Cr ratio in patients using benzodiazepines at the time of the scan was 0.15 (SD = 0.05) as compared to 0.14 (SD = 0.04) in patients not using benzodiazepines at the time of the scan. In fact, the four patients with the lowest prefrontal GABA/Cr ratios did not use benzodiazepines at the time of the scan.

Correlations between GABA and glutamate

There was no significant correlation between prefrontal GABA/Cr ratio and prefrontal glutamate in the patients (r(13)=-0.38, p=n.s.) and in the controls (r(14)=-0.15, p=n.s.). Correlations were based on the unstandardized for age, sex, and gray and white matter fraction over the whole group. The findings did not alter when leaving out the corrections for gray and white matter fractions. In the occipital cortex a significant correlation was found between GABA/Cr ratio with glutamate level in the controls (r(10)=-0.56, p=0.03), with higher GABA/Cr ratios being associated with lower levels of glutamate, which was not significant in the patients (r(9)=-0.25, p=n.s.). There was no significant correlation between prefrontal and occipital GABA/Cr ratios in the patients (r(13)=-0.38, p=n.s.) and in the controls (r(14)=-0.15, p=n.s.).

Discussion

To our knowledge, this study presents the first proton magnetic resonance spectroscopy measurements of GABA and glutamate levels *in vivo* in the brains of patients with schizophrenia at a magnetic field strength of 7T. The main finding is a significantly lower prefrontal GABA/Cr ratio in patients with schizophrenia as compared to healthy controls. The lower prefrontal GABA/Cr ratio was strongly associated with level of general cognitive functioning. No significant change in the GABA/Cr ratio was found between patients and controls in the occipital cortex. No significant changes between levels of glutamate, NAA, creatine, and choline between patients and controls were found in the prefrontal and occipital cortex.

The main finding of this study is a significantly lower GABA/Cr ratio in the prefrontal cortex in patients as compared to controls. This finding is consistent with postmortem studies that were done in the brains of patients with schizophrenia. Decreased levels of mRNA encoding for GAD67, the enzyme that facilitates GABA synthesis from glutamate, were found in postmortem brain tissue, which suggest diminished GABA production [12-15]. Decreased levels of mRNA encoding for GAT-1, the transporter that removes GABA from the synaptic cleft, suggest enhanced GABAergic neurotransmission, however, decreased levels of mRNA encoding for GAT-1 might also reflect a mechanism to compensate for reduced GABA synthesis [18;19]. In addition, increased expression of the $\alpha 2$ subunits of the $GABA_A$ receptor, the major inhibitory receptor in the brain mediating most of the physiological actions of GABA [20], may be a mechanism to compensate reduced GABA synthesis as well. On the other hand, decreased expression of the of the $\alpha 1$, $\alpha 5$, γ and δ receptor subunits is not likely to serve as a compensation for diminished GABA levels and might be a primary disease process which may decrease GABAergic neurotransmission, eventually resulting in reduced GABA levels [21]. Overall, studies suggest a net reduction of GABA levels in schizophrenia, which is consistent with our finding that in the prefrontal and not in the occipital cortex there is a significant decreased GABA/Cr ratio in patients with schizophrenia. Three healthy subjects had GABA/Cr ratio levels that were with >0.18, the highest levels that we measured in our subjects. When these 3 subjects were left out of the study the main effect of GABA/Cr level in the prefrontal cortex between groups was no longer significant. However, these levels are within normal limits based on statistical analysis of our data, and based on data from earlier studies in healthy subjects that were within the same limits. Therefore we think that this effect may indeed represent a decrease in GABA/Cr ratios in schizophrenia as compared to controls. However, future studies with larger numbers of subjects should confirm our findings at 7T before we can

we can make a more definite conclusion regarding decreases in prefrontal cortex GABA concentrations in schizophrenia.

Interestingly, we find the decrease in GABA/Cr ratios in patients with schizophrenia to be strongly associated with their general level of cognitive functioning (r=-0.91, p=0.001). In patients, the association between higher general level of intelligence and lower GABA/Cr ratio was found for all aspects of intelligence. When separated out for several aspects of IQ, the decreased GABA/Cr ratio in patients was highly associated with higher working memory functioning, followed by perceptual reasoning, and verbal comprehension (all ≥ 0.70). As observed in many earlier studies, we also found a lower intelligence level in patients with schizophrenia, which possibly exists prior to the disease onset [45] and is associated with genetic risk for schizophrenia [46]. Unexpectedly, the most prominent decrease in GABA/Cr ratio as compared to healthy controls was found in those patients that functioned above average intelligence level despite their illness. Possibly, this finding may be explained by results from studies in the non-human brain. GABA is involved in learning and memory in both mammals and insects. Reducing GABA synthesis in the anterior paired lateral (APL) neuron in *Drosophila* results in enhanced learning, thus suggesting an antagonistic relationship between GABA and (olfactory) learning and memory [47]. Since we find this negative association in patients and not in healthy controls, this could reflect a compensatory mechanism to continue functioning at a (above) average level. Such a compensatory mechanism may possibly explain that more pronounced decreased GABA/Cr levels were associated with less negative symptom severity in patients. Recently, rare exonic deletions were found in schizophrenia as well as other neurodevelopmental conditions, implicating the synaptic organizer Gephyrin (GPHN) that is responsible for the clustering and localization of GABA receptors at inhibitory synapses [48]. Increased levels of Gephyrin have been associated with cognitive impairment in an animal study [49]. Thus, although we can only speculate at this point, our finding of decreased GABA/Cr levels in the prefrontal cortex, particularly in high functioning patients with schizophrenia, may reflect either a cognitive functioning-associated genetic risk factor for the disease, or a compensatory mechanism to continue functioning at the (above) average level.We found no significant changes in glutamate levels in patients with schizophrenia as compared to healthy controls. A recent meta-analysis showed an overall reduction of frontal glutamate levels in patients as compared to healthy controls [3]. However, in that same study it was also suggested that glutamate levels are increased in the early stage of the disease and begin to decrease below healthy control levels around the age of 25 years when most patients have been ill for a few years. Since the

average age of the schizophrenia patients in this study is 27.6 years with average illness duration of 77 months (**Table 1**), it is possible that glutamate levels in these patients are overall approaching healthy control levels because of their current disease stage. Other levels of brain metabolites, including those of prefrontal and occipital NAA, a marker of neuronal integrity, and of creatine, a marker of energy metabolism, did not differ significantly between patients and healthy controls. This is in contrast with a meta-analysis showing reduced NAA levels in patients with schizophrenia in the frontal lobe [50]. However, most of these studies were in patients who had been ill for many years, and their reduction of NAA (and of creatine) levels might be due to progressive brain volume reductions [1;2;50].

With increasing age, and irrespective of disease, we find, significantly lower occipital GABA, and prefrontal glutamate, NAA, and creatine levels in the current 7T ^{1}H-MRS study. Occipital but not frontal GABA levels decreased with increasing age in these relatively young adult individuals, whereas for all the other metabolites the frontal but not occipital levels were lower with older age. Possibly, high levels of the inhibitory neurotransmitter GABA are still important for development and plasticity of the prefrontal cortex in young adulthood [51] . Decreases in glutamate level are consistent with previous studies [3;52], and may be associated with age-dependent neuronal pruning [52;53]. Lower NAA levels with increasing age have been reported before [54-56] and may reflect a reduction of brain tissue or neuronal function. A lower creatine level with increasing age may be a sign of maturation of neural systems in the brain [51]. With normal aging, brain volume reduces [1;57], which may result in less pronounced reductions of NAA and creatine levels. More likely, the decreasing levels reflect different aspects of neural systems integrity and energy. Thus, overall, there were significant lower levels of all metabolites with older age, particularly in the prefrontal and not in the occipital cortex, with the exception of GABA with had lower levels in occipital but not prefrontal cortex with increasing age. We found no significant differences in the extent of decline with age in patients with schizophrenia as compared to controls, although some levels (NAA) did seem to decline disproportional in the patients.

Significant differences between the sexes were found in prefrontal glutamate and occipital NAA levels, with males having lower levels as compared to females, irrespective of disease. We did not find any differential findings for males and females in patients as compared to controls. This is in contrast to a previous MRS study on metabolite changes and sex differences in chronic schizophrenia showing lower NAA levels in the basal ganglia in male subjects as compared to female subjects, and decreased ACC glutamate, NAA and creatine levels in male patients but not in female patients [58]. Indeed, morphological changes in schizophrenia are

more prominent in male patients, and males also show a greater vulnerability to schizophrenia [58-60]. However, in the current study, our findings of GABA/Cr differences between patients and controls could not be explained by differences between the sexes.

In vivo MRS measurements have some limitations to take into account. One, with MRS one cannot distinguish between intracellular and extracellular metabolite levels. Two, because of its low concentration a large voxel size is needed to reliably and time-efficiently measure GABA. Hence the voxel contained both gray and white matter. However, the gray and white matter fractions in the individual voxels were accounted for in the measurement of the metabolite levels and were controlled for in the analyses making a large influence on the findings unlikely. Also, the findings did not alter considerable when leaving out the corrections for gray and white matter fractions from the analyses. Another important limitation of this study is the lack of control for antipsychotic medication use, since antipsychotics may alter metabolite levels in the brain [61]. While most associations between antipsychotic use and metabolite levels did not reveal significant associations, we did find a negative correlation between prefrontal GABA/Cr ratios and current daily dosage of olanzapine, and a positive correlation between occipital NAA levels and current daily dosage of clozapine. Indeed, antipsychotic medication may reduce GABA and Glx (the sum of glutamate and glutamine) levels in the medial prefrontal cortex [26]. Intake of benzodiazepines may increase GABA levels, since benzodiazepines are $GABA_A$ receptor agonists [62] (but see [63] for a decrease in GABA levels by down-modulation of GAD function and gene expression). While we found no significant associations with cumulative or current intake, we did observe that patients that were currently taking benzodiazepines have higher GABA/Cr ratios compared to those who did not. Indeed two-thirds of the patients had used benzodiazepines since they had become ill and one-third of the patients were currently taking benzodiazepines.

In conclusion, using ^{1}H-MRS at 7T, prefrontal GABA/Cr ratios appear to be decreased in patients with schizophrenia as compared to healthy controls. Moreover, GABA/Cr ratios are strongly associated with the level of cognitive functioning, with high functioning patients having lower GABA/Cr ratios. This suggests a role for GABA in the earlier stages of the disease in schizophrenia, and further studies are needed to assess the effects of antipsychotic medication use, aging, and symptomatology.

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7

Intellectual ability and brain metabolites

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Abstract

Intelligence is a measure of general cognitive functioning capturing a wide variety of different cognitive functions. It has been hypothesized that the brain works to minimize the resources allocated towards higher cognitive functioning. Thus, for the intelligent brain it may be that not simply more is better, but rather more efficient is better. In both glutamatergic and GABAergic neurons, the primary energetic costs are associated with neurotransmission. We tested the hypothesis that minimizing energy resources is beneficial to intelligence in the prefrontal and occipital cortices by measuring GABA and glutamate levels at 7T ^{1}H-MRS in healthy individuals. In this study we therefore use a GABA to glutamate ratio as an index for efficiency of energy use. We find that working memory index is positively associated with GABA/Cr to glutamate ratio in the occipital cortex in individuals. Moreover, higher Performance IQ and Perceptual Reasoning Index were positively associated with NAA – a putative marker for neuronal integrity - in the prefrontal cortex. Thus, it seems that individuals with a higher intelligent working memory performance make more efficient use of their brains' energy resources.

Introduction

Intelligence is a measure of general cognitive functioning capturing a wide variety of different cognitive functions [1]. Intelligence has long been (albeit modestly) associated with brain size [2-4]. More recently, intellectual functioning has been implicated in brain functioning [5], and in the efficiency of the functional [6] and structural brain network [7]. Regional structural differences in relationship to intelligence have been demonstrated in several studies in healthy individuals [2;3;8], and in individuals with local brain lesions [9]. However, it is not known how the human brain handles complex cognitive tasks while being such an expensive organ to operate, utilizing some 20% of all oxygen taken in and 25% of all glucose produced while representing only about 2% of the body's weight [10;11]. Indeed, it has been hypothesized that the brain works to minimize the resources allocated towards higher cognitive functioning [12]. Thus, for the intelligent brain it may be that not simply more is better, but rather more *efficient* is better.

Glutamate (Glu) and GABA (gamma-aminobutyric acid) are the major excitatory and inhibitory neurotransmitters in the central nervous system (CNS). In both glutamatergic and GABAergic neurons, the primary energetic costs are associated with neurotransmission, and the energetic needs of these neurons dominate the cerebral cortex energy requirements [13;14]. In the resting awake state, 80% of energy used by the brain supports events associated with neuronal firing and cycling of GABA and glutamate, and in the actively awake individual, the change in energy (and its coupled activity) induced by stimulation during task performance is very small in comparison to its baseline value [15]. Thus, minimizing resources through the inhibition-excitation balance encompassing GABA and glutamate in the resting-state brain may be beneficial to general cognitive functioning. While general intellectual functioning has been related to brain neurochemistry in several studies, measuring largely positive associations with the brain metabolite N-acetyl aspartate (NAA) [16;17], a marker of neuronal integrity [18;19], these measures do not provide information on the brain's energy use. To obtain such information one needs to reliably measure both glutamate and GABA levels, which is not an easy task using MRI scanners operating at conventional magnetic field strengths.

Performing ^{1}H-MRS at ultra-high magnetic field strength (7T) has two clear advantages. The sensitivity of the measurements of metabolite concentrations is increased because of the increased signal-to-noise ratio (SNR) and the increased spectral resolution allows for a better separation of the individual metabolite spectra. For instance, at a magnetic field strength of 7T it is now possible to adequately separate the glutamate and glutamine signals resulting in a higher

accuracy of glutamate measurement [20]. However, detection of metabolites at a higher magnetic field strength is complicated by reduced accuracy in localization of the region of interest [21;22]. The sLASER (semi-localized by adiabatic selective refocusing) sequence enables ^{1}H-MRS of the human brain at 7T with increased localization accuracy, SNR and reliability [21;23]. Despite the increased SNR the measurement of GABA is not straightforward because the concentration of GABA is low compared to other brain metabolites and the GABA signal is obscured by other overlapping metabolites with higher signal intensity. To overcome this problem, spectral editing techniques can be applied to isolate the GABA signal [24]. In this study we used the sLASER sequence combined with editing techniques (MEGA-sLASER) which allows for accurate and time-efficient detection of GABA [22].

We tested the hypothesis that minimizing energy resources is beneficial to intelligence in the prefrontal and occipital cortices, both known for their implications in intelligence [25], by measuring GABA and glutamate levels using ^{1}H-MRS at a magnetic field strength of 7T in healthy adults. A combination of higher GABA levels and lower glutamate levels (i.e. more inhibitory activity and less excitatory activity) suggests a more efficient energy use [13]. In this study we therefore use a GABA to glutamate ratio as an index for efficiency of energy use.

Methods

Subjects

A total of 23 healthy individuals (16 males/7 females) participated in the study. Participants had no major psychiatric or neurological history, no history of drug or alcohol abuse, and no first-degree relatives with psychiatric or neurological disorders. The study was approved by the Medical Ethics Committee of the University Medical Center Utrecht, The Netherlands, and performed according to the directives of the Declaration of Helsinki (amendment of Seoul, 2008). Participants provided written informed consent prior to the examination. Mean (SD) age was 27.7 (5.3) years, and average completed years of education was 16 (min-max = 11-18 years).

Cognitive assessment

All participants underwent a general cognitive assessment using the full Wechsler Adult Intelligence Scale (WAIS–III-NL) [26]. The Total Intelligence Quotient (TIQ) as well as the Verbal (VIQ) and Performance (PIQ) Intelligence Quotients, the Perceptual Reasoning Index (PRI), Verbal Comprehension Index (VCI) and the Working Memory Index (WMI) were measured.

MR acquisition

All investigations were performed on a 7T whole body MR scanner (Philips, Cleveland, OH, USA). A birdcage transmit head coil was used in dual transmit driven by 2x4 kW amplifiers, in combination with a 32-channel receive coil (both Nova Medical, Inc., Burlington, MA, USA).

For anatomical reference and gray and white matter tissue classification a T_1-weighted magnetization prepared rapid gradient echo (MP-RAGE) sequence was obtained (450 slices, slice thickness = 0.8 mm, TR = 7 ms, TE = 3 ms, flip angle = 8 degrees, FOV = 250 x 200 x 180 mm, 312 x 312 acquisition matrix, SENSE factor 2.7, scan duration = 408 s).

For the assessment of glutamate and the major spectral components NAA, creatine and choline, ^{1}H-MRS experiments were conducted using a sLASER sequence (semi-localized by adiabatic selective refocusing; TE = 28 ms, 32 averages, TR = 5 s) (**Figure 1A**). Voxels (2 x 2 x 2 cm^3) were located in the medial prefrontal and medial occipital lobe (**Figure 2**). Non-water-suppressed spectra were obtained for quantification (carrier frequency was set to the chemical shift of H_2O, acquisition time = 10 s).

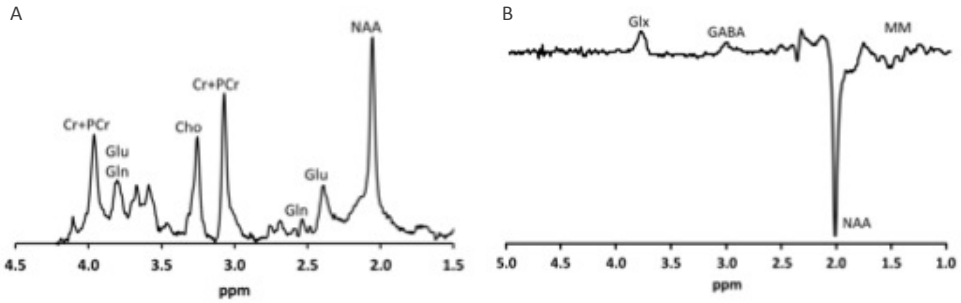

Figure 1: Typical metabolite spectra (A) as recorded using the sLASER sequence and (B) as recorded using the MEGA-sLASER sequence.

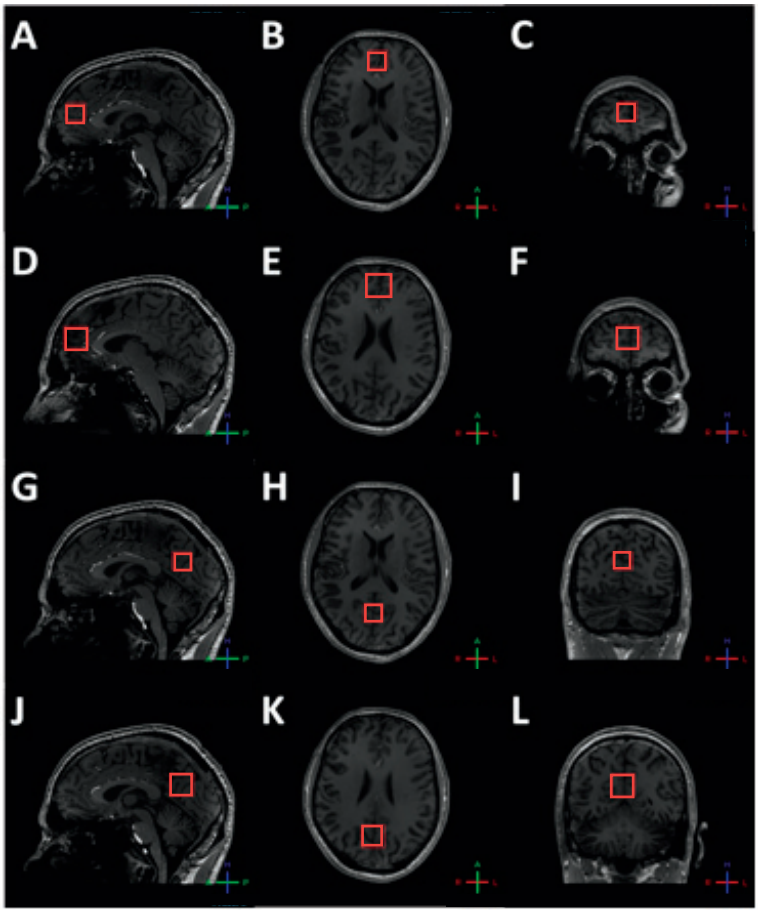

Figure 2: Voxel placement. A: frontal sLASER voxel, sagittal view; B: frontal sLASER voxel, axial view; C: frontal sLASER voxel, coronal view; D: frontal MEGA-sLASER voxel, sagittal view; E: frontal MEGA-sLASER voxel, axial view; F: frontal MEGA-sLASER voxel, coronal view; G: occipital sLASER voxel, sagittal view; H: occipital sLASER voxel, axial view; I: occipital sLASER voxel, coronal view; J: occipital MEGA-sLASER voxel, sagittal view; K: occipital MEGA-sLASER voxel, axial view; L: occipital MEGA-sLASER voxel, coronal view.

GABA-edited ^{1}H-MRS experiments were conducted using a MEGA-sLASER sequence (TE = 74 ms, 64 averages, TR = 4 s) (**Figure 1B**). Voxels (2.5 x 2.5 x 2.5 cm^3) were located in the medial frontal and medial occipital region (**Figure 2**). Prior to the MRS exams, second order B_0 shimming was applied using the FASTERMAP algorithm at the voxel of interest [27;28]. Second, at this location, a high B_1 field was generated to minimize chemical shift displacement artefacts [29]. The highest possible B_1 field was generated by optimizing the phase of both transmit channels to locally assure constructive B_1 interferences [21].

Spectral fitting and quantification

Retrospective phase and frequency alignment was performed on all data sets of each measurement [30]. Fitting of the sLASER spectra was performed with LCModel-based software implemented in Matlab [31], which uses a priori knowledge of the spectral components to fit metabolite resonances [32]. The following 16 metabolites and a measured macromolecular baseline [33] were fitted to the spectra: acetate, aspartate, choline (Cho), phosphorylcholine (PC), glycerophosphorylcholine (GPC), phosphorylethanolamine (PE), creatine (Cr), phosphocreatine (PCr), N-acetyl aspartate (NAA), N-acetyl aspartyl glutamate (NAAG), GABA, Glu, glutamine (Gln), glutathione (GSH), myo-inositol (mIns), and taurine (Tau). Levels of Glu, total NAA (NAA+NAAG), total creatine (Cr+PCr) and total choline (Cho+PC+GPC+PE) were estimated using the water signal as an internal reference and calculated as follows:

$$[\mathrm{met}] = \left(\frac{\frac{\mathrm{signal}_{\mathrm{met}}}{\mathrm{signal}_{\mathrm{water}}} * \left(\mathrm{volGM} * [\mathrm{water}_{\mathrm{GM}}] + \mathrm{volWM} * [\mathrm{water}_{\mathrm{WM}}] + \mathrm{volCSF} * [\mathrm{water}_{\mathrm{pure}}] \right)}{\mathrm{volGM} + \mathrm{volWM}} \right)$$

Where [met] is the metabolite concentration, $\mathrm{signal}_{\mathrm{met}}$ is the fitted signal intensity of the metabolite, accounting for the number of protons, and $\mathrm{signal}_{\mathrm{water}}$ is the fitted signal intensity of water, accounting for the number of protons; volGM, volWM and volCSF are respectively the gray matter fraction, white matter fraction and cerebrospinal fluid (CSF) fraction in the voxel; and [$\mathrm{water}_{\mathrm{GM}}$], [$\mathrm{water}_{\mathrm{WM}}$], and [$\mathrm{water}_{\mathrm{pure}}$] are respectively the water concentration in gray matter, white matter or CSF. For determining the contribution of gray matter, white matter and CSF of each voxel, the software package SPM8 was used to segment the T_1-weighted image. In the T_1-weighted image, the position of the ^{1}H-MRS voxel was determined, after which the amount of gray matter, white matter and CSF in the ^{1}H-MRS voxel was computed. To account for differences in transverse relaxation between water and metabolites, a correction was applied based on reported T_2 values at 7T of 47 ms on average for water and 107 ms assumed for the metabolites [34]. Statistical analysis of the gray and white matter fractions in the frontal and occipital MEGA-sLASER (GABA/Cr) and sLASER (glutamate, NAA, creatine, choline) revealed correlations >0.95 for both gray and white matter fractions in the two voxels.

Fitting of the MEGA-sLASER spectra was performed by frequency-domain fitting of the GABA and creatine resonances to a Lorentzian line-shape function in Matlab. GABA levels were expressed as the ratios of their peak areas relative to the peak areas of the creatine resonance.

Because of poor spectral quality as established by a Cramér-Rao lower bound (CRLB) of more than 20% and visual inspection, some data were excluded from the

study. Frontal MRS results are based on 18 subjects and occipital MRS results are based on 17 subjects. Frontal GABA-edited MRS results are based on 19 subjects and occipital GABA-edited MRS results are also based on 19 subjects.

Statistical analysis

Statistical analyses were performed using SPSS 21.0 (2012, Chicago, IL). Data were controlled for their normality of the distributions. No transformations for correction were needed. To evaluate differences in metabolite concentrations and gray and white matter fractions between the frontal and occipital areas paired t-tests were done. To evaluate associations between brain metabolite levels with general intelligence measures, Pearson correlation coefficients were done with corrections for age, sex and for gray and white matter fractions.

Results

Intelligence measures

The mean (SD) of the Total Intelligence Quotient (TIQ) was 108 (13), the Verbal Intelligence Quotient (VIQ) was 109 (12), the Performance Intelligence Quotient (PIQ) was 107 (14), the Verbal Comprehension Index (VCI) was 111 (13), the Perceptual Reasoning Index (PRI) was 108 (15), and the Working Memory Index (WMI) was 105 (12) (**Table 1**)

Metabolite concentrations in the frontal and occipital areas

Paired t-tests for differences in metabolite concentrations and gray and white matter fractions between the frontal and occipital areas revealed overall somewhat higher metabolite concentrations in the occipital as compared to the frontal area (except for choline, for which the concentration was lower). However, after correction for gray and white matter fraction in the voxel the differences in NAA and choline concentrations between these regions were no longer significant ($p>.8$).

Table 1: Intelligence and brain metabolites in healthy individuals*.

Intelligence	(Sub) test score	(Mean (SD))	Min	Max
	Total IQ	108 (13)	82	131
	Verbal IQ	109 (12)	82	128
	Performance IQ	107 (14)	78	127
	Verbal Comprehension Index	111 (13)	91	132
	Perceptual Reasoning Index	108 (15)	79	129
	Working Memory Index	105 (12)	86	124
1H-MRS sequence	**Metabolite**	**Prefrontal (Mean (SD))**	**Occipital (Mean (SD))**	**Paired t-test**
MEGA-sLASER	GABA/Cr ratio	.12 (.02)	.15 (.04)	ns
	Gray matter (%)	68.1 (11.3)	68.0 (11.9)	ns
	White matter (%)	24.0 (11.8)	27.7 (13.2)	ns
sLASER	Glutamate (mM)	12.11 (1.91)	12.26 (1.54)	ns
	NAA (mM)	13.37 (1.75)	14.79 (1.80)	p<.05
	Creatine (mM)	11.93 (2.08)	12.12 (1.77)	ns
	Choline (mM)	7.17 (2.81)	4.97 (1.60)	p<.01
	Gray matter (%)	71.4 (14.3)	70.1 (12.7)	ns
	White matter (%)	19.5 (15.6)	25.2 (14.6)	p<.05

* Uncorrected data based on two separate ^{1}H-MRS measurements and T1w volume measurements performed in two brain areas (prefrontal and occipital). For assessment of GABA/Cr ratios a MEGA-sLASER sequence was performed and successfully completed in 19 individuals in the medial prefrontal cortex and in 18 individuals in the medial occipital cortex. A sLASER sequence was performed for assessment of glutamate, NAA, creatine, and choline levels and successfully completed in 18 individuals in the medial prefrontal cortex and in 17 individuals in the medial occipital cortex. Volume data are based on 21 individuals except for the occipital MEGA-sLASER voxel volumes, which are based on 18 individuals.

Brain metabolites, gray and white matter fractions, and intelligence

A higher Working Memory Index was associated with a significantly lower glutamate concentration (r(10)=-.79, p<0.004) and with a higher (but not significant) GABA/Cr ratio (r(9)=.42, p=0.19), resulting in a significantly higher GABA/Cr to glutamate ratio in the occipital cortex (r(7)=.73, p=0.04) (**Table 2; Figure 3**). A higher Working Memory Index was also associated with a lower glutamate concentration in the frontal cortex (r(10)=-.53, p=0.076), and with a lower creatine level in the prefrontal cortex (r(10)=-.46, p=.13), but these findings did not reach statistical significance. Moreover, Performance Intelligence Quotient and Perceptual Reasoning Index were positively associated with N-acetyl-aspartate concentration in the frontal cortex (both with r(10)=.56, p=.056) (**Table 2; Figure 4**). No significant associations were found for Total Intelligence Quotient, Verbal Intelligence Quotient, and Verbal Comprehension Index. All correlations with metabolites were corrected for age, sex, and gray and white matter fractions.

In addition, there were significant positive associations between a higher prefrontal

gray matter fraction and Verbal Intelligence Quotient (r(15)=.54, p=0.02), and Verbal Comprehension Index (r(15)=.53, p=0.03). A positive association between prefrontal gray matter fraction and Total Intelligence Quotient (r(15)=.41, p=.11) and a negative association between prefrontal white matter fraction and Verbal Intelligence Quotient (r(15)=.43, p=0.08) did not reach statistical significance. These correlations were corrected for age and sex.

Table 2: Brain metabolites and intelligence[1]

	Cognition					
Metabolites	TIQ	VIQ	PIQ	VCI	PRI	WMI
Prefrontal						
GABA/Cr	.31	.28	.30	.25	.37	-.05
glutamate	-.01	-.25	.26	.01	.28	-.53''
N-acetyl aspartate	-.26	-.02	.56"	.29	.56"	-.28
creatine	-.25	-.43	.01	-.20	-.08	-.46'
choline	-.21	-.11	-.33	-.13	-.45	-.18
Occipital						
GABA/Cr	.03	.09	-.08	.04	-.18	.42'
glutamate	-.19	-.27	-.07	-.17	-.10	-.79**
N-Acetyl-Aspartate	-.00	-.11	.11	-.02	-.02	-.42
creatine	-.09	-.16	-.06	-.29	-.18	-.21
choline	-.17	-.15	-.20	.11	-.27	-.26

[1]) Pearson correlations, corrected for age, sex, and for local gray matter and white matter fractions.
**= Significant association at p<.01 (occipital glutamate with WMI p=.004); ''=p<.10 (prefrontal NAA with PIQ and prefrontal NAA with PRI p=0.056; prefrontal glutamate with WMI p=0.076); '=p<.25 (prefrontal creatine with WMI p=.13 occipital GABA/Cr with WMI p=.21).
TIQ= Total intelligence quotient; VIQ=verbal intelligence quotient; PIQ=performance intelligence quotient; VCI=verbal comprehension index; PRI=perceptual reasoning index; WMI=working memory Index.

Brain metabolites, age and sex

A higher age was associated with a lower prefrontal creatine concentration (r(12)=-.52, p=0.045), and occipital creatine concentration (r(12)=-.56, p=0.04). Prefrontal glutamate concentration (r(12)=-.35, p=0.20) did also decline with increasing age but this finding did not reach statistical significance. There were no other significant associations between metabolite levels in the prefrontal and occipital cortices with age. These correlations were corrected for sex, and gray and white matter fractions. There were no significant associations between metabolite levels in the prefrontal and occipital cortices with sex.

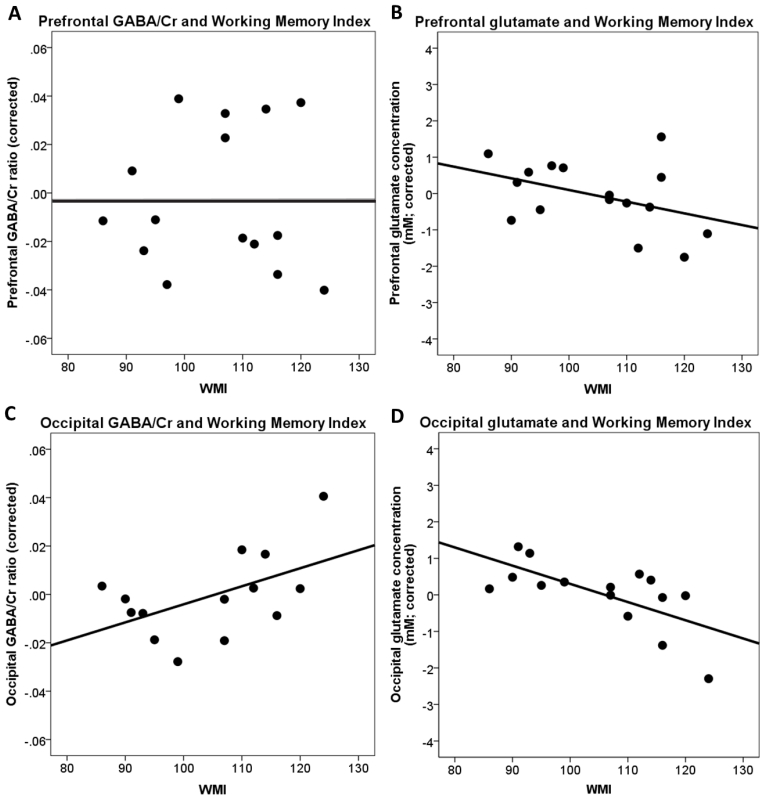

Figure 3. Correlations between prefrontal and occipital GABA/Cr and glutamate with Working Memory Index (WMI). (A) Prefrontal GABA/Cr ratios are not significantly correlated with WMI; (B) Prefrontal glutamate concentrations are not significantly correlated with WMI; (C) Occipital GABA/Cr ratios are not significantly correlated with WMI; (D) Occipital glutamate concentrations are significantly correlated with WMI ($r(10)=-0.79$, $p<0.004$). Data are presented corrected for gray and white matter fractions, age, and sex.

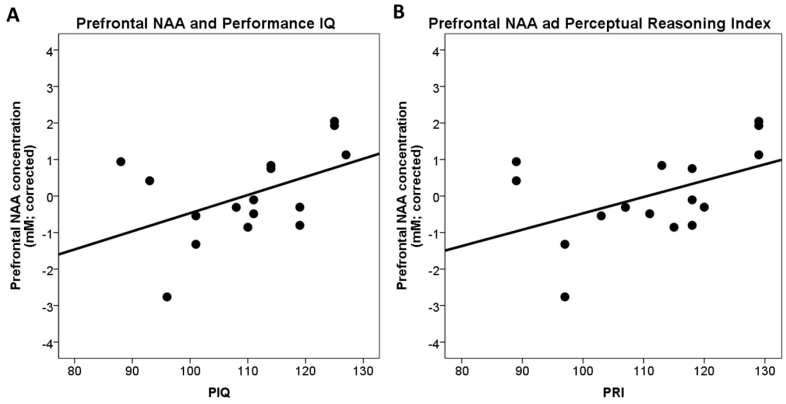

Figure 4. Correlations between prefrontal NAA with (A) Performance Intelligence Quotient (PIQ) and (B) Perceptual Reasoning Index (PRI) (both $r(10)=0.56$, $p=0.056$) . Data are presented corrected for gray and white matter fractions, age, and sex.

Correlations among brain metabolites, and gray and white matter fractions

Correlations between metabolite levels were more prominent within the occipital than within the prefrontal cortex, while uncorrected for their gray and white matter fractions and corrected for age and sex (**Table 3**).

Following correction for age, sex, gray and white matter fractions, in the prefrontal cortex a significant negative association between GABA/Cr ratios and creatine ($r(11)=-.56$, $p<0.05$) concentrations, and a significant positive association between glutamate and NAA ($r(7)=.73$, $p<0.01$) and between glutamate and creatine ($r(12)=.53$, $p<0.05$) concentrations were found (**Table 3; Figure 5**). In the occipital cortex a significant negative association between GABA/Cr ratios and glutamate ($r(7)=-.85$, $p<0.01$) concentrations, and a significant positive association between NAA and creatine ($r(11)=.55$, $p<0.05$) concentrations were found.

Higher GABA/Cr ratios in the prefrontal cortex were significantly correlated with lower GABA/Cr ratios ($r(7)=-.68$, $p<0.05$), and lower glutamate concentrations ($r(7)=-.89$ $p<0.01$) in the occipital cortex. Correlations were corrected for their respective gray and white matter fractions.

In the occipital cortex a higher fraction of gray matter and a lower fraction of white matter correlated with overall higher metabolite levels. In the prefrontal cortex this was less obvious, revealing one significant negative correlation between gray matter fraction and NAA level. High positive correlations between the frontal and occipital regions for gray and white matter fractions and high negative correlations for gray versus white matter within and between these regions were found. These correlations were corrected for age and sex.

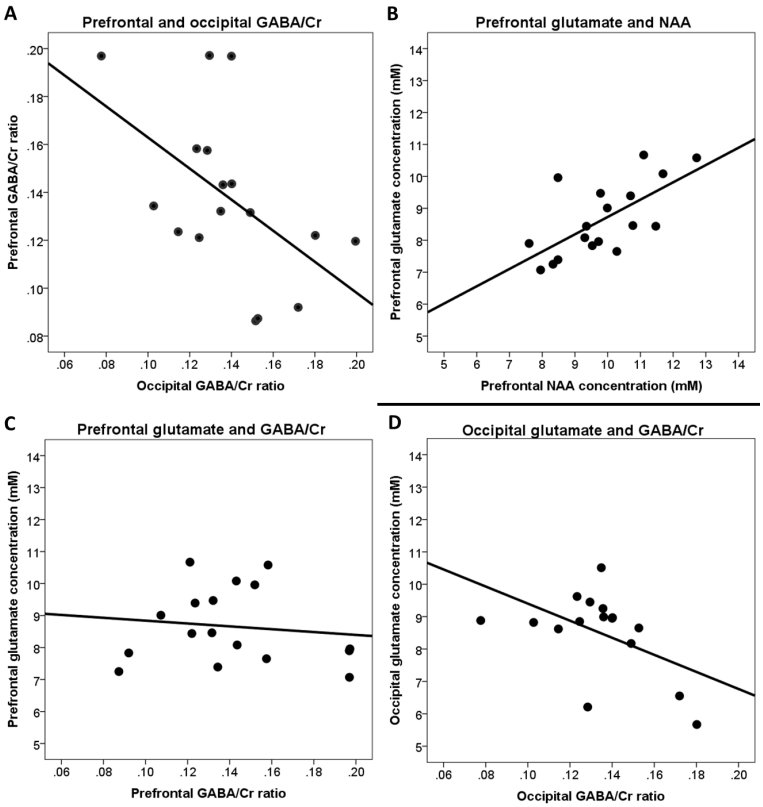

Figure 5: Correlations between brain metabolites in the prefrontal and occipital cortices. (A) Prefrontal and occipital GABA/Cr ratios are significantly correlated ($r(7)=-0.68$, $p<0.05$); (B) Prefrontal NAA and glutamate concentrations are significantly correlated ($r(7)=0.73$, $p<0.01$); (C) Prefrontal GABA/Cr ratios and glutamate concentrations are not significantly correlated; (D) Occipital GABA/Cr ratios and glutamate concentrations are significantly correlated ($r(7)=-0.85$, $p<0.01$). Data are presented uncorrected for gray and white matter fractions, age, and sex.

Discussion

To our knowledge, this study presents the first proton magnetic resonance spectroscopy measurements of GABA and glutamate levels *in vivo* at a magnetic field strength of 7T associated with level of intelligence. The main finding is that a higher Working Memory Index is associated with a significantly higher GABA/Glu ratio in the frontal and occipital cortices. In addition, performance IQ and perceptual reasoning index were positively associated with NAA in the prefrontal cortex.

Our main finding is that a lower glutamate in the frontal and occipital cortices and a higher GABA/Cr level in the occipital cortex are significantly associated with a higher Working Memory Index in healthy individuals. This suggests that more

Table 3. Brain metabolites in the prefrontal and occipital corticwes

^{1}H-MRS	Frontal							Occipital						
Frontal	GABA/Cr	Glu	NAA	Cr	Cho	GM	WM	GABA/Cr	Glu	NAA	Cr	Cho	GM	WM
GABA/Cr	-	.11	-.18	-.46”	-.12	-.01	-.19	-.50”	.44	.18	.16	.27	.24	.33
Glu	-.01	-	.66**	.56*	.08	-.11	-.10	-.27	.23	.27	.03	.07	-.01	-.11
NAA	-.25	.73**	-	.54”	-.08	-.50*	.40	.13	-.15	.30	-.31	-.11	-.41	.32
Cr	-.61*	.53*	.50”	-	.45	-.23	.10	-.16	.04	.30	.10	-.05	-.17	.12
Cho	-.12	.10	.02	.41	-	.19	-.17	-.07	.10	.05	.10	.50”	.08	-.02
GM	-	-	-	-	-	-	-.84**	-.36	.38	-.03	.58**	.40	.76**	-.62**
WM	-	-	-	-	-	-	-	.42	-.57*	-.31	-.73**	-.52*	-.81**	.81**
Occipital														
GABA/Cr	-.68*	-.89**	-.65”	-.61”	-.11	-	-	-	-.67**	-.01	-.26	-.26	-.33	-.34
Glu	.32	.20	.08	-.10	.12	-	-	-.85**	-	-.54*	.71**	.55*	.62*	-.67**
NAA	-.39	.07	.42	.30	.14	-	-	.14	.35	-	.58*	.25	.30	-.46”
Cr	-.25	-.10	-.01	.43	.13	-	-	-.31	.37	.55*	-	.48”	.84**	-.86**
Cho	.28	.29	.21	.08	.50	-	-	-.26	.32	.11	.03	-	.55*	-.53*
GM	-	-	-	-	-	-	-	-	-	-	-	-	-	-.96**
WM	-	-	-	-	-	-	-	-	-	-	-	-	-	-

**=p<0.01; *=p<0.05; “=p<0.10
Top right reflects Pearson correlations corrected for age and sex; Bottom left reflects Pearson correlations corrected for age, sex and for the respective gray and white matter fractions for GABA/Cr and the other metabolites in the frontal and occipital cortices.

efficient energy use in these parts of the brain is beneficial for working memory. Working memory refers to the ability to actively hold information on-line over brief periods of time [35]. Working memory is one of the factors marking intelligence, and has been closely related to general intelligence, although the extent of overlap is point of discussion [36]. Moreover, working memory has been positively associated with gray and white matter volume, and with white matter tracts, and these associations are under genetic control [3;37]. Interestingly, based on studies in animals it has been found that a successful working memory performance requires an exquisite balance of the excitatory and inhibitory circuitry in the prefrontal cortex that includes glutamate and GABA [35]. Neurons in the prefrontal cortex have been shown to fire persistently during the maintenance phase of working memory tasks for which a balance between inhibitory and excitatory neurons are thought to be required. Supportive evidence for such a system during activation in the human brain was found in a study where a higher resting GABA concentration was associated with higher amplitude of the BOLD fMRI response in the visual cortex [38] (for review see [39]). Other positive associations were found between resting-state GABA concentration in the occipital cortex with orientation discrimination performance [40] (see [41] for a negative finding), and in the supplementary motor area with tactile discrimination performance [42], which also support associations between GABA concentrations in the human cortex with cognitive functioning. Here we show, by using ^{1}H-MRS at 7T, that higher resting GABA/Cr ratios and lower glutamate concentrations may contribute to a more efficient balance leading to a higher working memory performance in healthy adults.

We also found that NAA was positively correlated with performance IQ and perceptual reasoning in the prefrontal cortex. This positive association is consistent with earlier findings at lower field strength where the NAA peak is also prominently visible, and was found to be positively associated with performance IQ [16]. In that particular study NAA was quantified, as we did in our study - in contrast to earlier studies were often NAA/Cr ratios were reported and the contribution of NAA as compared to Cr could not be clearly determined. NAA is turned over within neurons through a complex exchange between neurons and oligodendrocytes [43]. The rate of synthesis of NAA has been tightly coupled with glucose metabolism (Moreno et al, 2001). Low levels of NAA have been associated with brain disorders [18]. Indeed, this positive finding strengthens the idea that metabolite levels during the resting state may predict individual differences in cognitive functioning in humans.

Our measurements of brain metabolites were done in the frontal and occipital cortices using sLASER and MEGA-sLASER at 7T. To which extend were the metabolites correlated within individuals? The most prominent positive correlation

was found between levels of NAA with glutamate in the prefrontal cortex (.73). Other correlations between metabolite levels in the prefrontal cortex were a positive correlation of creatine with glutamate, and a negative correlation of creatine with GABA/Cr. The most prominent negative association was found between levels of glutamate and GABA/Cr ratio in the occipital cortex (-.85). A positive correlation in the occipital cortex was found between levels of NAA and creatine. Interestingly, significant negative correlations between GABA level in the occipital cortex with GABA level in the prefrontal cortex and with glutamate level in the prefrontal cortex were also found, thus suggesting a possible differential and maybe connected resting state association with GABA between these two anatomically distant brain regions. It also means that our findings of metabolite levels with cognitive functioning are not working in isolation but are part of a network of connective metabolites.

In vivo MRS measurements have some limitations to take into account. One, with MRS one cannot distinguish intracellular and extracellular metabolite levels. Two, because of its low concentration a large voxel size is needed to reliably and time-efficiently measure GABA.

Future studies using instance modern network analyses [44] may reveal how such connections between metabolite levels act. Such studies may reveal to which extent the associations between intelligence and brain metabolites are linked to local glucose levels and BOLD fMRI effects as well as to network efficiency of these nodes with the rest of the brain. There is evidence that the metabolic costs of a brain area (i.e., a node in a network) are proportional to the number of (mathematical) paths it has to connect with other nodes, and that the metabolic costs of a path are proportional to the physical distance it spans between nodes [45]. Interestingly, Brodmann areas with a high glycolytic index were also found to be hub areas (i.e., have a high centrality rank); including Brodmann areas 32 and 33 [46] which are anatomically overlapping with the medial prefrontal voxel location of our current study. A functional neural network study revealed that a higher intelligence was associated with more efficient brain network [6], and thus possibly with a more efficient use of local brain metabolism including GABA and glutamate.

In conclusion, Working Memory Index is positively associated with the GABA to glutamate ratio in healthy individuals, suggesting that a more efficient energy use is beneficial for working memory performance. Moreover, higher Performance IQ and Perceptual Reasoning Index were positively associated with NAA – a putative marker for neuronal integrity - in the prefrontal cortex. Thus, it seems that individuals with a higher intelligent working memory performance make more efficient use of their brains' energy resources.

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8

Summary and discussion

Proton magnetic resonance spectroscopy provides a powerful tool to non-invasively investigate brain metabolism *in vivo,* which can be of particular interest when studying glutamate and GABA levels in patients with schizophrenia. Glutamate and GABA are likely to be reduced in schizophrenia. Moreover, alterations in glutamate and GABA levels might explain the cognitive deficits that are commonly seen in schizophrenia. This thesis describes ^{1}H-MRS findings of especially the major excitatory neurotransmitter glutamate and the major inhibitory neurotransmitter GABA in schizophrenia and health. Experiments were conducted using an ultra-high magnetic field of 7T, whereas most of the ^{1}H-MRS studies of schizophrenia until now used lower magnetic field strengths. The major advantage of measuring metabolite concentrations at ultra-high magnetic field strength is that the resonance peaks of glutamate and glutamine can be adequately separated, thereby facilitating a reliable measurement of glutamate.

Glutamate in schizophrenia

Chapter 2 describes a meta-analysis of ^{1}H-MRS studies of glutamate in schizophrenia until 2011. A total of 24 studies in individuals at high risk for psychosis, first-episode patients and chronically ill patients were analyzed for changes in glutamate and its precursor glutamine. In the frontal brain region, glutamate appeared to be lower in patients as compared to healthy controls and glutamine appeared to be higher in patients as compared to healthy controls. Moreover, both glutamate and glutamine appeared to decrease progressively with age in patients with schizophrenia as compared to healthy controls. This is supported by the fact that the glutamine-glutamate ratio is increased in patients and decreases with age. This ratio is most prominently increased in younger patients and seems to normalize with aging, possibly reflecting a glutaminase -the enzyme that converts glutamine into glutamate- deficiency resulting in decreased glutamate and increased glutamine levels [1]. Glutamine levels in patients drop below healthy control levels around the age of 35 years, so elevations in glutamine might possibly be an early marker for glutamate changes in schizophrenia. The decreasing levels of NAA, a marker of neuronal integrity, that were also found in this meta-analysis may be associated with progressive reductions of brain volume observed in earlier studies in schizophrenia [2-7]. However, the decrease in glutamate may at least partly but not solely be explained by a decrease in brain volume. The altered glutamate levels in schizophrenia could be explained by diminished activation of the NMDA receptor, causing reduced synaptic activity. Indeed, administration of NMDA antagonists leads to decreased glutamate and increased glutamine levels [8-10].

NMDA receptor hypofunction results in a decline of excitatory neuronal activity, thereby influencing the monitoring function of GABAergic inhibitory neurons which may in turn downregulate their activity [11;12].

Since important alterations in glutamatergic neurotransmission in schizophrenia appear to take place in young adulthood, investigating the course of glutamate levels in healthy individuals in that age range might elucidate the role of glutamate in schizophrenia. In **chapter 4**, a study on glutamate changes in the frontal cortex in healthy young adulthood is described. Glutamate levels decreased between 18 and 31 years of age, which could not be explained by altered brain tissue volumes or neuronal function. Thus, changes in glutamate may precede brain volume changes or may reflect the cortical changes that occur during young adolescence. During the transition from adolescence to adulthood, synaptic pruning occurs and mainly glutamatergic synapses are eliminated [13-15]. The changes in glutamate levels are likely to be caused by altered glutamatergic neurotransmission and metabolism, such as changes in glutaminase [16;17] and NMDA receptor density [18]. Additionally, both glutamate [19-21] and brain network efficiency [22-25] are associated with cognitive functioning, so the decline in glutamate levels observed in healthy young adults may reflect brain maturation. Thus, aberrant glutamate levels in young adults with schizophrenia may suggest anomalies in synaptic pruning and brain maturation.

In **chapter 6**, a 7T ^{1}H-MRS study in schizophrenia is presented, which showed no changes in glutamate levels, in both prefrontal and occipital cortices, in patients as compared to healthy controls. Since the average age of the patients in this study was 27.6 years, it may be possible that their glutamate levels approached healthy control levels because of their current disease stage, as the meta-analysis discussed in **chapter 2** showed that glutamate levels in patients start to decrease below healthy control levels around the age of 25 years. Irrespective of disease state, an age-related decline in glutamate levels was found in the prefrontal cortex, which is consistent with the meta-analysis presented in **chapter 2** and the study on glutamate levels in young adulthood in **chapter 4**. Also, lower prefrontal glutamate levels were found in males as compared to females, which might agree with earlier observations that morphological changes in schizophrenia are more prominent in males than in females and male show a greater vulnerability to schizophrenia [26-28].

GABA in schizophrenia

Chapter 3 reviews findings of postmortem and ^{1}H-MRS studies on GABA in schizophrenia. One of the most consistent findings is a reduction of the levels of GAD67 mRNA in patients with schizophrenia [29;30], which appears to be a component in the pathophysiology rather than a consequence of the illness [31], and results in reduced synthesis of GABA. This occurs predominantly in parvalbumin-positive GABAergic neurons [32] targeting the upregulated $\alpha 2$ subunit of the $GABA_A$ receptor at pyramidal neurons [33]. Furthermore, levels of mRNA encoding for the transporter protein GAT-1 are reduced, which is also related to the disease process and not attributable to other factors associated with schizophrenia, and results in reduced reuptake of GABA in presynaptic neurons [34-36]. Thereby, GABA neurotransmission at the synapse is improved, possibly to compensate for the deficit in GABA synthesis [37]. Similarly, upregulation of postsynaptic GABA receptors may compensate for the diminished GABA synthesis and also appears to be a disease trait [33;37]. The alterations in GABAergic neurotransmission were mainly found in the DLPFC, but since schizophrenia is characterized by various symptoms involving different brain regions (e.g. cognitive impairments and abnormalities in motor and sensory functioning) anomalies were also found in the anterior cingulate cortex, primary visual cortex, primary motor cortex, orbital frontal cortex, superior temporal gyrus, striatum and thalamus [38]. This may suggest that the overall reduced GABAergic activity is the consequence of a common upstream mechanism that operates across multiple cortical areas. However, earlier ^{1}H-MRS studies on GABA in schizophrenia are scarce and inconclusive.

Chapter 6 presents a 7T ^{1}H-MRS study in schizophrenia, which indeed found lower prefrontal GABA levels in patients as compared to healthy controls. Moreover, the decreased GABA levels in patients are strongly associated with their general level of cognitive functioning, the association between higher cognitive functioning and lower GABA level was found for all aspects of intelligence. The most prominent decrease in GABA levels was observed in patients that had an above average intelligence level, which may reflect a compensatory mechanism to continue functioning at this level [39-41]. In contrast to other metabolites, GABA did not show any age-related decreases in the relatively young subjects in this study, which may be due to the possible role of GABA in brain development and plasticity in young adulthood [42].

As discussed in **chapter 7**, lower glutamate and higher GABA levels in the occipital cortex, hence less excitatory and more inhibitory activity, are associated with a higher working memory index in healthy individuals, suggesting a less and

more efficient energy use in this part of the brain is beneficial for working memory.

^{1}H-MRS at 7T in the human brain *in vivo*

In **chapter 5**, the reproducibility of glutamate measurement in the human brain *in vivo* using ^{1}H-MRS at a magnetic field strength of 7T was assessed. As compared to the commonly used STEAM sequence, the newly developed sLASER sequence for 7T seems to be a more robust method for determining glutamate levels, particularly in the frontal brain region which plays an important role in schizophrenia. The variance in sLASER measurements were mainly caused by physiological differences between subjects, which is specifically important when studying populations that are difficult to recruit since sLASER requires a smaller sample size to detect physiological differences between groups. sLASER benefits from an increased signal-to-noise ratio and localization accuracy and reduced measurement time as compared to STEAM. Also, a fitting procedure including 12 or more metabolite basis sets seems to more robustly display metabolite levels [43;44]. Unfortunately, sLASER was not used in all experiments described in this thesis, since it only became available for use at 7T halfway through this project.

Methodological considerations

Several limitations have to be taken into account when using ^{1}H-MRS to study the human brain *in vivo*. With MRS, one cannot distinguish between intracellular and extracellular metabolite levels. Also, the glutamate signal that is detected does not distinguish between glutamate used in metabolic pathways and glutamate used for neurotransmission. Furthermore, because of its low concentration, a large voxel size is needed to reliably and time-efficiently measure GABA. These large voxels will not only contain gray matter but rather a mixture of gray and white matter tissue. However, gray and white matter fractions in the MEGA-sLASER as well as the sLASER voxels were accounted for in measurement of metabolite levels and were controlled for in the statistical analyses in **chapters 4-7**.

The meta-analysis described in **chapter 2** comprises several different studies, differences in paradigms, such as magnetic field strength, acquisition method, quantification method and frontal brain region have to be taken into account [45]. Also, not all of the included studies did take into account partial voluming effects, so the age-related decrease in glutamate levels that was observed may be at least partly due to brain volume changes [46],

Antipsychotics may alter metabolite levels in the brain [47;48]. In the meta-analysis as well as the 7T ^{1}H-MRS study in schizophrenia, there was no control for antipsychotic medication use. Indeed, a negative correlation was found between prefrontal GABA levels and current daily dosage of olanzapine and a positive correlation between occipital NAA levels and current daily dosage of clozapine. Intake of benzodiazepines may influence GABA levels; two-thirds of the patients had used benzodiazepines before and one-third of the patients were on benzodiazepines at the time of the scan. Benzodiazepines are $GABA_A$ receptor agonists and may thus increase GABA levels. Indeed, patients that used benzodiazepines at the time of the scan showed slightly (but not significantly) higher GABA levels as compared to patients that did not use benzodiazepines at the time of the scan. On the other hand, benzodiazepines may down-modulate GAD function and gene expression, which may partly explain the decreased prefrontal GABA levels that were found in patients. Although these correlations could not explain the main findings reported in **chapter 6**, future studies including never-medicated schizophrenia patients are needed to confirm these findings without medication use as a confounding factor.

Future directions

Up until now, studies investigating glutamate and GABA in schizophrenia using ^{1}H-MRS *in vivo* were mostly cross-sectional. Longitudinal studies are essential when investigating glutamate and GABA levels during the course of the disease. Since the meta-analysis presented in **chapter 2** suggested that glutamate levels are upregulated at a young age in patients with schizophrenia and individuals at risk for psychosis, examining adolescents and young adults at high risk for psychosis and first-episode patients may elucidate the mechanisms behind the development of the disease. Also, adequate control for medication intake, duration of illness and severity of symptoms is needed.

Conclusion

Glutamate and GABA can be successfully assessed in the human brain *in vivo* using ^{1}H-MRS at 7T, particularly by using the sLASER sequence. Because of the enhanced resolution, ^{1}H-MRS at 7T increases the number of detectable metabolites and provides a more accurate measurement of metabolites.

According to previous research, glutamate levels decline progressively with age in the frontal brain region in patients with schizophrenia. Especially young

adulthood appears to be a critical period in schizophrenia, both in terms of glutamatergic alterations and cognitive functioning. In healthy young adults, glutamate levels decline with age, which is likely to be due to brain maturation. Hence, glutamatergic anomalies in this age range suggest a disturbed brain maturation. The 7T ^{1}H-MRS study presented in this thesis describes that GABA levels decrease in the prefrontal brain region in patients with schizophrenia, suggesting a loss of synaptic activity that does not seem to be caused by a loss of brain volume. The reduction of prefrontal GABA levels in schizophrenia *in vivo* is in line with evidence from earlier postmortem studies. In this relatively young sample, occipital but not prefrontal GABA levels decrease with age irrespective of disease whereas all other metabolites show decreasing prefrontal but not occipital levels with increasing age, indicating an important role for GABA in the development of the prefrontal cortex. Moreover, the decline of prefrontal GABA levels in patients is strongly associated with their general level of cognitive functioning. While glutamate levels are not decreased in this group of patients as compared to the healthy volunteers, probably because of their relatively young age, they do decline with age irrespective of disease in the prefrontal region, which is again consistent with existing literature. Interestingly, both changes in glutamatergic as well as GABAergic neurotransmission appear to be a pathophysiological component of schizophrenia, rather than a consequence of the disease.

Furthermore, a higher working memory index, a marker for intelligence, in healthy subjects is associated with lower glutamate and higher GABA levels in the occipital cortex. Since most of the brain's energy is used for glutamatergic and GABAergic activity, minimizing resources through the excitation-inhibition balance may be beneficial to cognitive functioning. Lower glutamatergic -excitatory- acitivity and higher GABAergic -inhibitory- activity indicates that high working memory performance is related to a more efficient use of the brain's energy resources. Disturbances in the GABA-glutamate ratio, as observed in schizophrenia in this thesis, may thus point to a less efficient use of the brain's energy resources.

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9

Nederlandse samenvatting

Proton magnetische resonantie spectroscopie (^{1}H-MRS) biedt de mogelijkheid om het metabolisme in de hersenen *in vivo* en non-invasief te meten. Dit kan met name interessant zijn bij het bestuderen van glutamaat- en GABA-concentraties bij patiënten met schizofrenie. Het is aannemelijk dat glutamaat- en GABA-concentraties verminderd zijn bij schizofrenie. Daarbij kunnen verandeirngen in glutamaat- en GABA-concentraties mogelijk de cognitieve afwijkingen verklaren die vaak voorkomen bij schizofrenie. Dit proefschrift beschrijft in het bijzonder ^{1}H-MRS bevindingen van de belangrijkste stimulerende neurotransmitter glutamaat en de belangrijkste remmende neurotranmitter GABA bij schizofrenie en in gezondheid. Hierbij is gebruik gemaakt van een ultrahoog magnetisch veld van 7 tesla (T), terwijl de meeste ^{1}H-MRS schizofrenieonderzoeken tot nu toe van lagere magnetische veldsterktes gebruik maakten. Het grootste voordeel van het meten van metabolietconcentraties op een ultrahoge magnetische veldsterkte is dat de resonantiepieken van glutamaat en glutamine adequaat van elkaar onderscheiden kunnen worden, waardoor een betrouwbare meting van glutamaat mogelijk is.

Glutamaat bij schizofrenie

Hoofdstuk 2 beschrijft een meta-analyse van ^{1}H-MRS studies naar glutamaat bij schizofrenie tot 2011. In totaal zijn 24 studies naar individuen met een hoog risico voor psychose, patiënten met een eerst episode en chronisch zieke patiënten geanalyseerd op veranderingen van glutamaat en glutamine, de precursor van glutamaat. In de frontaalkwab lijkt de glutamaatconcentratie lager te zijn bij patiënten ten opzichte van gezonde controles en de glutamineconcentratie lijkt hoger te zijn. Bovendien lijken zowel glutamaat- als glutamineconcentraties progressief af te nemen met leeftijd bij patiënten ten opzichte van gezonde controles. Dit wordt ondersteund door het feit dat de glutamine-glutamaatratio verhoogd is bij patiënten en afneemt met leeftijd. De verhoging van deze ratio is het meest opvallend bij jongere patiënten en normaliseert schijnbaar met het ouder worden, wat mogelijk een deficiëntie van glutaminase -het enzym dat glutamine omzet in glutamaat- weergeeft waardoor glutamaatconcentraties afnemen en glutamineconcentraties toenemen [1]. Bij patiënten komen de glutamineconcentraties onder het niveau van gezonde controles rond het 35^{e} levensjaar, verhogingen van glutamineconcentraties kunnen dus een vroege indicatie zijn van veranderingen bij schizofrenie. Afnemende concentraties NAA, een indicator van neuronale integriteit, die ook zijn gevonden in deze meta-analyse kunnen geassocieerd zijn met de progressive afname van hersenvolume, wat geobserveerd is in eerdere studies [2-7]. Echter, de afname van glutamaatconcentraties kan slechts gedeeltelijk,

maar niet geheel, verklaard worden door een afname van hersenvolume. De veranderde glutamaatconcentraties bij schizofrenie kunnen verklaard worden door verminderde activatie van de NMDA-receptor, waardoor de synaptische activiteit afneemt. Uit eerdere studies is inderdaad gebleken dat toediening van NMDA-antagonisten leidt tot een afname van glutamaatconcentraties en een toename van glutamineconcentraties [8-10]. Hypofunctie van de NMDA-receptoren resulteert in een afname van stimulerende neuronale activiteit, waarbij de controlerende functie van GABAerge remmende neuronen wordt beïnvloed en de activiteit van deze neuronen kan afnemen [11;12].

Omdat belangrijke veranderingen in glutamaterge neurotransmissie bij schizofrenie lijken plaats te vinden in jonge volwassenheid, zou het onderzoeken van het verloop van glutamaatconcentraties bij gezonde jongvolwassenen de rol van glutamaat bij schizofrenie kunnen ophelderen. In **hoofdstuk 4** wordt een studie naar glutamaatveranderingen in de frontale hersenschors bij gezonde jongvolwassenen beschreven. Glutamaatconcentraties namen af tussen het 18e en 31e levensjaar, wat niet verklaard kon worden door een verandering in hersenvolume of neuronaal functioneren. De veranderingen in glutamaatconcentraties kunnen dus veranderingen in hersenvolumes voorafgaan of geven mogelijk de corticale veranderingen weer die optreden tijdens jonge adolescentie. Gedurende de overgang van adolescentie naar volwassenheid neemt het aantal synaptische verbindingen af -een proces dat *synaptic pruning* wordt genoemd- met name het aantal glutamaterge synapsen wordt gereduceerd [13-15]. De veranderingen in glutamaatconcentraties worden waarschijnlijk veroorzaakt door een veranderde glutamaterge neurotransmissie en metabolisme, door bijvoorbeeld veranderingen in glutaminase [16-17] en NMDA-receptordichtheid [18]. Daarbij komt dat zowel glutamaat [19-21] en de efficiëntie van netwerken in de hersenen [22-25] geassocieerd zijn met cognitief functioneren, dus de afname van glutamaatconcentraties bij gezonde jongvolwassenen is mogelijk een reflectie van de rijping van de hersenen. Afwijkende glutamaatconcentraties bij jongvolwassenen met schizofrenie kunnen dus anomalieën in *synaptic pruning* en maturatie van de hersenen weergeven.

In **hoofdstuk 6** wordt een 7T ^{1}H-MRS studie bij schizofrenie gepresenteerd, waarin geen veranderingen in glutamaatconcentraties, zowel in de frontale als occipitale hersenschors, zijn aangetoond bij patiënten in vergelijking met gezonde controles. Omdat de gemiddelde leeftijd van de patiënten 27.6 jaar was, kan het wellicht zijn dat hun glutamaatconcentraties het niveau van de gezonde controles benaderden gezien hun huidige ziektefase. De meta-analyse in **hoofdstuk 2** toonde immers aan dat glutamaatconcentraties bij patiënten onder het niveau

van gezonde controles komen rond het 25e levensjaar. Ongeacht ziektestatus is er een afname van glutamaatconcentraties in de prefrontale hersenschors geobserveerd, wat overeenkomt met de meta-analyse in **hoofdstuk 2** en de studie naar glutamaatconcentraties bij jongvolwassenen in **hoofdstuk 4**. Ook zijn er lagere prefrontale glutamaatconcentraties gevonden bij mannen ten opzichte van vrouwen, wat in overeenstemming is met eerdere studies waaruit is gebleken dat de morfologische veranderingen die geobserveerd worden bij schizofrenie prominenter aanwezig zijn bij mannen en en dat mannen kwetsbaarder zijn dan vrouwen voor het ontwikkelen van schziofrenie [26-28].

GABA bij schizofrenie

Hoofdstuk 3 geeft een overzicht van de bevindingen van postmortem en ^{1}H-MRS studies naar GABA bij schizofrenie. Een van de mest consistente bevindingen is een afname van de concentratie GAD67 mRNA bij patiënten met schizofrenie [29;30], wat eerder een component van de pathophysiologie dan een gevolg van de ziekte lijkt te zijn [31], en wat resulteert in gereduceerde GABA-synthese. Dit komt voornamelijk voor in parvalbumine-positieve GABAerge neuronen [32], die projecteren op het verhoogde aantal α 2 subeenheden van de $GABA_A$-receptoren op pyramidale neuronen. Verder zijn de concentraties van mRNA dat codeert voor het transporteiwit GAT-1 gereduceerd, wat eveneens gerelateerd is aan het ziekteproces en niet te wijten is aan andere factoren die geassocieerd zijn met schizofrenie, en resulteert in een afname van heropname van GABA in presynaptische neuronen [34-36]. Daardoor wordt de GABAerge neurotransmissie in de synaps verbeterd, mogelijk ter compensatie van het tekort aan GABA-synthese [37]. De verhoogde expressie van postsynaptische GABA-receptoren dient mogelijkerwijs ook als compensatie van de afgenomen GABA-synthese en lijkt ook een eigenschap van de ziekte te zijn [33;37]. Veranderingen in GABAerge neurotransmissie zijn hoofdzakelijk gevonden in de dorsolaterale prefrontale cortex, maar omdat schizofrenie gekarakteriseerd wordt door verschillende symptomen die betrekking hebben op verschillende hersengebieden (bijvoorbeeld verslechtering van cognitieve vermogens en afwijkingen in motorisch en sensorisch functioneren) zijn er ook anomalieën gevonden in de cortex cingularis anterior, primaire visuele cortex, primaire motorcortex, orbitofrontale cortex, superieure temporale gyrus, het striatum en de thalamus [38]. Dit suggereert wellicht dat de over het algemeen afgenomen GABAerge activiteit het gevolg is van een gemeenschappelijk mechanisme dat in meerdere corticale gebieden actief is. Echter, eerdere ^{1}H-MRS studies naar GABA bij schizofrenie zijn schaars en de uitkomsten ervan blijven onbeslist.

In **hoofdstuk 6** wordt een 7T ^{1}H-MRS naar schizofrenie beschreven, waarbij lagere prefrontale GABA-concentraties bij patiënten werden gevonden ten opzichte van gezonde controles. Bovendien zijn de afgenomen GABA-concentraties bij patiënten sterk geassocieerd met het algemene cognitieve niveau; de associatie tussen hoger cognitief functioneren en lagere GABA-concentraties werd gevonden voor alle aspecten van intelligentie. De meest prominente afname van GABA-concentraties werd gezien bij patiënten met een bovengemiddelde intelligentie, wat mogelijk een compensatoir mechanisme weergeeft om op dit niveau te blijven functioneren [39-41]. In tegenstelling tot andere metabolieten werden in deze relatief jonge populatie geen leeftijdsgebonden afnames van GABA-concentraties geobserveerd, wat te maken kan hebben met de mogelijke rol van GABA bij de ontwikkeling en plasticiteit van de hersenen in jongvolwassenheid [42].

Zoals wordt besproken in **hoofdstuk 7** zijn lagere glutamaat- en hogere GABA-concentraties in de occipitale hersenschors, dus minder stimulerende en meer remmende activiteit, geassocieerd met een beter werkgeheugen in gezonde individuen, wat suggereert dat minder en efficiënter energieverbruik in dit hersengebied voordelig is voor het functioneren van het werkgeheugen.

^{1}H-MRS op een veldsterkte van 7T in het menselijk brein *in vivo*

In **hoofdstuk 5** wordt de reproduceerbaarheid getest van glutamaatmetingen in het menselijk brein *in vivo*, waarbij gebruik wordt gemaakt van ^{1}H-MRS op een magnetische veldsterkte van 7T. Vergeleken met de veelgebruikte sequentie STEAM lijkt de recent ontwikkelde sLASER sequentie voor 7T een robuustere methode om glutamaatconcentraties te bepalen, met name in het frontale hersengebied dat een belangrijke rol speelt bij schizofrenie. De variantie in sLASER-metingen werd voornamelijk veroorzaakt door fysiologische verschillen tussen individuen, wat specifiek van belang is bij het bestuderen van populaties die moeilijk te werven zijn voor onderzoek omdat met sLASER een kleinere steekproef nodig is om fysiologische verschillen tussen groepen te detecteren. Ten opzichte van STEAM heeft sLASER het voordeel van een verhoogde signaal-ruis ratio, een nauwkeurigere localisatie en kortere scantijd. Hierbij geeft een fitting procedure met een basisset van 12 of meer metabolieten de metabolietconcentraties robuuster weer [43;44]. Helaas is sLASER niet gebruikt in alle experimenten omdat de sequentie pas halverwege dit project beschikbaar werd voor gebruik op 7T.

Methodologie

Verschillende beperkingen dienen in acht genomen te worden bij het gebruik van ^{1}H-MRS bij het onderzoeken van de hersenen *in vivo*. Met MRS kan geen onderscheid gemaakt worden tussen intracellulaire en extracellulaire metabolietconcentraties. Er is dus ook geen onderscheid tussen glutamaat dat gebruikt wordt voor metabolisme en glutamaat dat gebruikt wordt voor neurotransmissie. Verder is voor GABA, vanwege de lage concentratie ervan, een groot voxel nodig voor betrouwbare en efficiënte metingen. Deze grote voxels bevatten niet alleen grijze stof, maar ook witte stof. Bij het berekenen van metabolietconcentraties is gecorrigeerd voor zowel grijze en witte-stoffracties van de MEGA-sLASER als sLASER sequentie, hiervoor is ook gecontroleerd bij de statistische analyses in de **hoofdstukken 4 t/m 7**.

De meta-analyse in **hoofdstuk 2** bevat verschillende studies, er moet rekening gehouden worden met de verschillen in de gebruikte methode zoals magnetische veldsterkte, acquisitie, kwantificatie, en het specifieke hersengebeid dat onderzocht is [45]. Ook hebben niet alle studies rekening gehouden met grijze en witte-stoffracties in het voxel, dus de leeftijdsgebonden afname van glutamaatconcentraties die is gevonden kan tenminste gedeeltelijk verklaard worden door volumeveranderingen in het brein [46].

Antipsychotica kunnen invloed hebben op metabolietconcentraties in de hersenen [47;48]. Zowel bij de meta-analyse als de 7T ^{1}H-MRS studie bij schizofrenie is niet gecontroleerd voor antipsychoticagebruik. Er is wel een negatieve correlatie gevonden tussen prefrontale GABA-concentraties en de huidige dagelijkse dosis olanzapine en een positieve correlatie tussen occipitale NAA-concentraties en de huidige dagelijkse dosis clozapine. Gebruik van benzodiazepinen kan ook invloed hebben op GABA-concentraties; tweederde van de patiënten heeft ooit benzodiazepinen gebruikt en eenderde gebruikte benzodiazepinen ten tijde van het onderzoek. Benzodiazepinen zijn $GABA_A$-receptoragonisten en kunnen als zodanig GABA-concentraties verhogen. De patiënten die ten tijde van het onderzoek benzodiazepinen gebruikten vertoonden inderdaad licht (niet significant) verhoogde GABA-concentraties vergeleken met patiënten die geen benzodiazepinen gebruikten ten tijde van het onderzoek. Aan de andere kant kunnen benzodiazepinen de functie en genexpressie van GAD verlagen, wat -gedeeltelijk- de verlaagde prefrontale GABA-concentraties bij patiënten zou kunnen verklaren. Alhoewel deze correlaties de belangrijkste bevindingen in **hoofdstuk 6** niet konden verklaren, is onderzoek naar medicatienaïeve schizofreniepatiënten -om medicatie-effecten uit te sluiten- nodig voor het bevestigen van deze bevindingen.

Wat te doen in de toekomst

Tot nu toe zijn alle *in vivo* ^{1}H-MRS studies naar glutamaat- en GABA-concentraties cross-sectioneel. Longitudinale studies zijn essentieel bij het onderzoeken van glutamaat- en GABA_concentraties tijdens het verloop van de ziekte. De meta-analyse in **hoofdstuk 2** suggereert dat glutamaatconcentraties verhoogd zijn op jonge leeftijd bij schizofreniepatiënten en individuen met een hoog risico op psychose. Daarom kan het onderzoeken van adolescenten en jongvolwassenen met een hoog risico op psychose en eerste-episode patiënten de mechanismen achter het ontstaan van de ziekte ophelderen. Ook is adequate controle voor medicatie-inname, ziekteduur en ernst van de symptomen nodig.

Conclusie

Glutamaat en GABA kunnen succesvol *in vivo* gemeten worden in het menselijk brein met 7T ^{1}H-MRS, met name door gebruik te maken van sLASER. Vanwege de verbeterde resolutie is het aantal te detecteren metabolieten op 7T hoger en is een accurate meting mogelijk.

Volgens eerdere onderzoeken dalen glutamaatconcentraties progressief met leeftijd in het frontale hersengebied bij schizofreniepatiënten. In het bijzonder blijkt jongvolwassenheid een kritieke periode te zijn, zowel wat betreft veranderingen in glutamaatconcentraties als cognitief functioneren. Bij gezonde jongvolwassenen dalen de glutamaatconcentraties met de leeftijd, wat waarschijnlijk komt door maturatie van de hersenen. Daarom zijn afwijkingen in glutamaatconcentraties in deze periode wellicht een teken van verstoorde hersenontwikkeling. De 7T ^{1}H-MRS studie in dit proefschrift laat zien dat prefrontale GABA-concentraties ijn verlaagd bij schizofreniepatiënten, wat een verlies van synaptische activiteit impliceert die niet veroorzaakt wordt door een verlies van hersenvolume. De afname van prefrontale GABA-concentraties *in vivo* is in overeenstemming met de bevindingen van eerdere postmortem studies. In deze relatief jonge populatie nemen occipitale, maar niet prefrontale GABA-concentraties af met leeftijd, wat aangeeft dat GABA een belangrijke rol speelt bij de ontwikkeling van de prefrontale hersenschors. Bovendien is deze afname sterk gecorreleerd met het algemeen cognitief niveau. Ondanks het feit dat glutamaatconcentraties niet verlaagd zijn bij deze groep patiënten, waarschijnlijk vanwege hun jonge leeftijd, nemen deze wel af in het prefrontale hersengebied ongeacht ziekte, wat wederom overeenkomt met bestaande literatuur. Zowel veranderingen in glutamaterge als GABAerge neurotransmissie zijn hoogstwaarschijnlijk een pathofysiologische component van schizofrenie en zijn geen consequentie van de ziekte.

Verder is een hoge werkgeheugenindex, duidend op intelligentie, bij gezonde individuen geassocieerd met lagere glutamaat- en hogere GABA-concentraties in de occipitale hersenschors. Omdat de meeste energie in de hersenen wordt gebruikt voor glutamaterge en GABAerge activiteit is het minimaliseren van energieverbruik via de balans tussen stimuleren en remmen mogelijk voordelig voor het cognitief functioneren. Lagere glutamaterge -stimulerende- activiteit en hogere GABAerge -remmende- activiteit wijst erop dat hoge prestaties van het werkgeheugen gerelateerd zijn aan efficiënter energiegebruik in de hersenen. Verstoringen in de GABA-glutamaat ratio, zoals gevonden bij schizofrenie in dit proefschrift, kunnen dus duiden op een minder efficiënte energiegebruik in de hersenen.

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Curriculum vitae

Anouk Marsman was born on the 17th of May 1982 in Zwolle. After obtaining her grammar school diploma in 2000, she started studying Biology at the University of Groningen in 2001. During her studies she performed several research projects. At the Department of Animal Physiology of the University of Groningen she investigated the impact of maternal stress during gestation on the development of the metabolic syndrome in offspring, at the Department of Psychiatry of the University of Pennsylvania she studied the dynamics of slow wave activity after partial sleep deprivation and recovery sleep, and at the Institute of Cellular Medicine of Newcastle University she investigated the effects of second-generation antipsychotic medication on the development of type 2 diabetes mellitus. In 2008, she graduated cum laude and started her PhD research at the Brain Center Rudolf Magnus of the University Medical Center Utrecht. She primarily investigated the role of the major neurotransmitters glutamate and GABA in the healthy and schizophrenic brain using magnetic resonance spectroscopy at a field strength of 7 tesla. At the moment, she is working as a postdoctoral fellow at the Department of Radiology & Radiological Sciences of the Johns Hopkins University School of Medicine in Baltimore.

Anouk Marsman is geboren op 17 mei 1982 te Zwolle. Na het behalen van haar gymnasiumdiploma in 2000, begon zij in 2001 met de studie Biologie aan de Rijksuniversiteit Groningen. Tijdens haar studie heeft zij verschillende onderzoeken uitgevoerd. Bij de afdeling Dierfysiologie van de Rijksuniversiteit Groningen onderzocht zij de impact van maternale stress tijdens de gestatie op de ontwikkeling van het metabool syndroom bij nakomelingen, bij de afdeling Psychiatrie van de University of Pennsylvania bestudeerde zij de dynamiek van trage-hersengolfactiviteit na gedeeltelijke slaapdeprivatie en herstelslaap, en bij het Institute of Cellular Medicine van Newcastle University onderzocht zij de effecten van tweede generatie antipsychotica op de ontwikkeling van diabetes mellitus type 2. In 2008 behaalde zij haar bul cum laude en begon met haar promotieonderzoek aan het Hersencentrum Rudolf Magnus van het Universitair Medisch Centrum Utrecht. Zij onderzocht voornamelijk de rol van de belangrijke neurotransmitters glutamaat en GABA in het gezonde en schizofrene brein met behulp van magnetische resonantie spectroscopie bij een veldsterkte van 7 tesla. Op dit moment werkt zij als postdoctoraal onderzoeker bij de afdeling Radiologie & Radiologische Wetenschappen van de Johns Hopkins University School of Medicine in Baltimore.

Dankwoord

Dit proefschrift is tot stand gekomen met hulp en ondersteuning van verschillende mensen, die ik hier graag wil bedanken.

Mijn promotoren Hilleke Hulshoff Pol en Peter Luijten, de samenwerking was altijd prettig en de meetings motiverend. Jullie hebben het mogelijk gemaakt om op het snijvlak van twee disciplines te werken, wat een unieke en zeer leerzame ervaring is geweest.

Mijn co-promotoren René Mandl en Dennis Klomp, dankzij jullie is alles wat we van plan waren ook daadwerkelijk uitvoerbaar geworden en zijn alle studies met goed gevolg afgerond. En nog veel belangrijker, dankzij jullie besef ik dat je voor het doen van wetenschappelijk onderzoek moet beschikken over veel relativeringsvermogen en een goed gevoel voor humor.

Alle co-auteurs, in het bijzonder Martijn van den Heuvel, je hebt me fantastisch op weg geholpen; Vincent Boer, echt geweldig dat je altijd in bent voor prutsen met de scanner en Matlab-scripts; Marc Bohlken, superbedankt voor al je hulp bij de patiëntenstudie; en René Kahn, veel dank voor de snelle revisies en de altijd zeer bruikbare tips.

Zonder Paula Ywema was het onmogelijk geweest om de patiëntenstudie binnen een redelijk tijdsbestek af te ronden. Heel erg bedankt voor al je hulp bij het werven van onderzoeksdeelnemers, en voor alle gezelligheid bij het wekelijks overleg.

Sylvia Alberto en Jeanette Sopacua, jullie hebben altijd alles in goede banen geleid wat betreft planning, logistiek en administratie, wat niet altijd makkelijk was met twee totaal verschillende onderzoeksgroepen om rekening mee te houden.

Natalia Petridou and Sjoerd Stuit, it was very exciting to try and start with GABA-BOLD measurements and it was always good fun working together!

Christiaan Vinkers en Lotte Houtepen, heel gaaf dat jullie het aandurfden om met MRS te beginnen. Lotte, ik vond het erg leuk om je te begeleiden bij je eerste stappen in de MRS-wereld!

Mijn stagiaires Jeroen de Jonge en Shahnam Sharif, jullie hebben je enorm ingezet voor de patiëntenstudie, zonder jullie was de organisatie ervan niet zo voorspoedig verlopen. Jeroen, je hebt een prachtig paper afgeleverd!

Ook veel dank aan mijn (oud-) kamergenoten bij Psychiatrie Anna, Christian, Inge, Jiska, Marinka en Mireille; en bij de 7T-groep Esben, Hugo en Nolan, voor alle broodnodige kopjes koffie en discussies over meer en minder (vooral dat laatste) relevante zaken.

De (oud-) Psychiatriemedewerkers Antoin, Bas, Cédric, Caroline, Florian, Gayane, Heleen, Hugo, Janna Marie, Judy, Kelly, Mariët, Neeltje, Rachel, Remko, Thalia, Tjerk, Yumas; en (oud-) spectro's en 7T-ers Alessandro, Alex, Alexander, Anja, Anna, Astrid, Bart, Bertine, Catalina, Christine, Daniël, Fredy, Hanneke, Hans, Ilse, Irene, Jaco, Jannie, Jeroen, Joep, Mariska, Robin, Ronald, Tijl, Wouter, Wybe (ik vergeet ongetwijfeld een aantal mensen, mea maxima culpa), bedankt voor de prettige samenwerking en gezelligheid bij de talloze borrels!

Aleksandra, Anke, Joost, Pieter, Simon en Zsuzsika, we hebben een weergaloos Jump the FENS 2010 neergezet, ik heb een toptijd met jullie gehad!

Jobbe Holtes heeft dit boekje uiterst kundig vormgegeven. Jobbe, je was meteen enthousiast toen ik je hiervoor vroeg en je bent er zeer voortvarend en met veel creativiteit ingedoken, heel erg bedankt!

Ik ben heel blij met een aantal goede vrienden, die ik veel te weinig zie maar met wie het altijd ontzettend leuk is, en die met hun gezelligheid, ontspanning, support en belangstelling zeker een belangrijke bijdrage hebben geleverd. Sipke & Alie, Michiel & Jellie, Inge, Dennis & Nynke, jullie zijn super! Sipke en Michiel, heel fijn dat jullie mijn paranimfen willen zijn.

Natuurlijk mijn ouders, pap & mam, dit is onmogelijk in een paar zinnen te vatten, ik ben jullie erg dankbaar voor alle mentale en praktische steun, vertrouwen en betrokkenheid.

Vincent, je oneindige geduld en vermogen om mij van mijn werk te houden, wat best wel een prestatie is en meestal ook heel erg nodig, hebben mij er absoluut doorheen gesleept. Het wordt alleen nog maar leuker!