Reduced GABA concentration is associated with physical disability in progressive Multiple Sclerosis

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ABSTRACT

Neurodegeneration is thought to be the major cause of ongoing, irreversible disability in the progressive stages of Multiple Sclerosis (MS). Gamma-aminobutyric acid (GABA) is the principle inhibitory neurotransmitter in the brain, and is needed for normal brain function. The aims of this study were to investigate if GABA levels (i) are abnormal in secondary progressive (SP) MS patients when compared with healthy controls; and (ii) correlate with physical and cognitive performance in this patient population.

Thirty patients with SPMS and 17 healthy controls underwent single-voxel MEGA-PRESS MRS at 3T, to quantify GABA levels in the prefrontal cortex, right hippocampus and left sensorimotor cortex. All subjects were assessed clinically and underwent a cognitive assessment. Multiple linear regression models were used to compare differences in GABA concentrations between patients and controls adjusting for age, gender, and tissue fractions within each spectroscopic voxel. Regression was used to examine the relationships between the cognitive function and physical disability scores specific for these regions with GABA levels, adjusting for age, gender, and total N-acetyl-aspartate and Glutamine-Glutamate complex levels.

When compared with controls, patients performed significantly worse on all motor and sensory tests, and were cognitively impaired in processing speed and verbal memory. Patients had significantly lower GABA levels in the hippocampus (adjusted difference = -0.403mM, 95% confidence intervals (CIs) -0.792, -0.014, p=0.043) and sensorimotor cortex (adjusted difference = -0.385mM, 95% CIs -0.667, -0.104, p=0.009) compared with controls. In patients, reduced motor function in the right upper and lower limb was associated with lower GABA concentration in the sensorimotor cortex. Specifically for each unit decrease in

GABA levels (in mM), there was a predicted -10.86 (95% CIs -16.786, -4.482) decrease in grip strength (kg force) ($p<0.001$) and -8.74 (95% CIs -13.943, -3.015) decrease in muscle strength ($p<0.006$).

This study suggests that reduced GABA levels reflect pathological abnormalities that may play a role in determining physical disability. These abnormalities may include decreases in the pre- and post-synaptic components of the GABAergic neurotransmission and in the density of inhibitory neurons. Additionally, the reduced GABA concentration may contribute to the neurodegenerative process, since it may result in increased firing of axons, with consequent increased energy demands, which may lead to neuroaxonal degeneration and loss of the compensatory mechanisms that maintain motor function. This study supports the idea that modulation of GABA neurotransmission may be an important target for neuroprotection in MS.

Keywords: MRI; multiple sclerosis; disease progression; disability; gamma-aminobutyric acid (GABA)

Abbreviations: GABA = gamma-aminobutyric acid; EDSS = expanded disability Status Scale; PASAT = Paced Auditory Serial Addition Test;

INTRODUCTION

There is a need to understand the mechanisms of neurodegeneration in progressive MS (Fox *et al.*, 2012). Secondary Progressive (SPMS) develops after an initial RRMS course (typically 10-15 years), with or without acute exacerbations during the progressive course (Lublin *et al.*, 2014). This results in irreversible and continuous neurological decline. There is a decrease in the development of new inflammatory lesions, but disability accumulation continues, which is likely to be due to ongoing neuroaxonal loss, the mechanisms for which include degeneration of chronically demyelinated white matter axons (Trapp *et al.*, 1999) and progressive cortical demyelination (Kutzelnigg *et al.*, 2005). SPMS results in significant motor and cognitive impairment in affected patients. Cognitive dysfunction is common, with prevalence rates of between 43% and 70% (Langdon *et al.*, 2012) and affects information processing speed (Amato *et al.*, 2010), episodic memory and executive function (Strober *et al.*, 2009). The pathological processes underlying clinical disability in MS are complex, and include neuronal and glial changes, with associated structural and metabolic abnormalities; these abnormalities may be detected in vivo by metabolic and molecular imaging (Ciccarelli *et al.*, 2014). A recent study found that in MS patients, reduced concentrations of grey matter Glutamate, the main excitatory neurotransmitter of the human brain, correlated with worse memory function (Muhlert *et al.*, 2013), suggesting that abnormalities in the neurotransmitter pathways may play a role in neurodegeneration, which underpins disability in MS.

Gamma-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the brain (DeFelipe, 1993) and is produced from glutamate by L-glutamic acid decarboxylase (GAD) within GABAergic neurons (Chang *et al.*, 2003). GABA is then metabolised to succinic acid semialdehyde by GABA transaminase (GABA-T) and then to succinate, mainly within astrocytic mitochondria (Chang *et al.*, 2003). GABA is needed for normal brain function, synaptic plasticity, cortical adaptation and reorganisation (Stagg, 2014). Altered GABA

concentrations have been detected in a number of conditions, including increases and reductions observed in epilepsy (MacDonald *et al.*, 2011), and reductions in anxiety disorders (Durant *et al.*, 2010), schizophrenia (Reynolds *et al.*, 2002) and autism (Gaetz *et al.*, 2014). A number of studies in idiopathic generalised epilepsy found an increase in GABA levels in the occipital (Simister *et al.*, 2003) and frontal lobes (Chowdhury *et al.*, 2014) of patients when compared with controls. It has been reported that GABAergic inhibition is one of the mechanisms involved in use-dependent plasticity in the intact human motor cortex (Stagg *et al.*, 2011; Levy *et al.*, 2002; Floyer-Lea *et al.*, 2006; Butefisch *et al.*, 2000). Changes in GABA concentration in the sensorimotor cortex during motor learning have been demonstrated (Floyer-Lea *et al.*, 2006). One pilot study carried out in RRMS (Bhattacharyya *et al.*, 2013) found that reduced motor performance correlated with increased GABA levels in the sensorimotor cortex. The increased GABA concentration was also associated with increased motor activation on functional MRI. Despite limitations, these in vivo results suggest that cortical reorganisation or adaptation occurring in the sensorimotor cortex in patients with RRMS, as reflected by increased fMRI response, is linked with increased GABA levels, and is a possible compensatory mechanism that maintains motor function (Bhattacharyya *et al.*, 2013).

In vivo quantification of GABA using ^{1}H-MRS spectroscopy is challenging due to the spectral overlap of GABA with more abundant metabolites, such as N-acetyl-asparate (NAA) at 2 ppm, Creatine (Cr) at 3 ppm and glutamate (Glu) and glutamine (Gln) at 2.3 ppm (Puts and Edden, 2012). Spectral editing methods such as MEGA-PRESS (MEscher GArwood - Point RESolved Spectroscopy), have allowed the discrimination of GABA from these metabolites (Mescher *et al.*, 1998). The MEGA-PRESS sequence also allows quantification of total N-acetyl-aspartate (tNAA) and the Glutamine-Glutamate complex (Glx). tNAA is a well-established spectroscopic marker of neuronal loss and/or metabolic dysfunction (Moffett

et al., 2007) that is often reduced in central nervous system diseases including multiple sclerosis (MS) (Kirov *et al*., 2013; Achnichts *et al.*, 2013). We have reported lower levels of Glx in the spinal cord of primary progressive MS patients compared to healthy controls, and have suggested that these reflect dysfunction in the glutamatergic pathway and neuroaxonal loss (Abdel-Aziz *et al.*, 2015).

Thus, the finding of: (i) reduced concentrations of Glutamate, which is the precursor of GABA, as observed in post-mortem (Wegner *et al.*, 2006) and in vivo MRS studies in progressive MS (Sastre-Garriga *et al.*, 2005); (ii) reduced GABA-related gene transcripts and density of inhibitory neuronal processes in the motor cortex of autopsied MS brains (Dutta *et al.*, 2006); and (iii) impaired compensatory mechanisms occurring in SPMS compared with RRMS (Rocca *et al.*, 2005), lead us to hypothesise that GABA levels are reduced in SPMS patients when compared with healthy controls and that they correlate with increased clinical disability. In order to test these two hypotheses, we measured the concentrations of GABA in the prefrontal cortex, right hippocampus (involved in visual and verbal memory) and left sensorimotor cortex using MEGA-PRESS MRS. We investigated whether there was an association between memory function and GABA concentration in the grey matter regions of the prefrontal cortex and hippocampus, and between sensory and motor function and GABA concentration in the left sensorimotor cortex. We examined if these associations were independent of imaging measures of structural damage, such as those sensitive to axonal loss and demyelination, derived from the same areas, and of tNAA and Glx, obtained within the same spectroscopic voxels.

MATERIALS & METHODS

Subjects

Patients with a diagnosis of secondary progressive MS (SPMS) who were not taking any medication that affects the GABAergic systems (e.g. Baclofen), for a minimum of 6 months prior to the time of scanning, and with an Expanded Disability Status Scale (EDSS) of between 4.0 - 6.5, were recruited into the study. Healthy controls were also recruited. Written informed consent was obtained for participants in the study, which was approved by our local research ethics committee.

Cognitive Tests

Patients and controls were assessed using a range of cognitive tests. Speed of information processing was assessed using the Symbol-Digit Modalities Test (SDMT) (Lezak *et al*., 2004), and the 3-second Paced Auditory Serial Addition Test (PASAT) (Rao *et al*., 1990), for which z-scores were obtained with reference to published norms (Fischer *et al*., 1999). Executive function was measured using the Stroop colour-word interference test (Trenerry *et al*., 1989) and Hayling sentence completion test (Burgess and Shallice., 1996). Verbal memory was assessed using The California Verbal Learning Test – II (CVLT-II) for immediate and delayed recall (Delis *et al*., 2000) and visuospatial memory was assessed using the Brief Visuospatial Memory Test Revised (BVMT-R) (Benedict *et al*., 1997). Working memory was assessed using the digit span from the Wechsler Adult Intelligence Scale-III (Wechsler *et al*., 1997). Premorbid IQ was measured using the National Adult Reading Test (Nelson, 1982). Levels of anxiety and depression were measured using with the Hospital Anxiety and Depression Scale (Zigmond *et al*., 1983).

Failure of a test (SDMT, Stroop, PASAT, Hayling Sentence Completion, Digit Span, CVLT-II and BVMT-R), was defined as a score of two or more SDs below the mean of the controls. Patients with significant cognitive impairment were defined as those showing failure on at least two tests.

Clinical Assessments

All patients were assessed using the Expanded Disability Status Scale (EDSS) (Kurtzke, 1983). All patients and controls were also assessed using the 9-Hole Peg Test (9-HPT) (Goodkin *et al*., 1988), Timed 25-Foot Walk Test (TWT) (Cutter *et al*., 1999), and the Medical Research Council (MRC) scoring system for muscle strength of the right upper and lower limb (Medical Research Council, 1943; Dyck *et al*., 2005). Z-scores were calculated for the 9-HPT and TWT from normative values displayed in the National Multiple Sclerosis Society Task Force database (Fischer *et al*., 1999). Mean grip strength from the right upper limb was measured using the Jamar hydraulic dynamometer (Sammons Preston. Incorporated, Bolingbrook, IL, USA) (Svens and Lee, 2005). The average of two trials for the TWT and the average of two trials for the 9-HPT were calculated (Fischer *et al*., 1999). Vibration perception thresholds (VPTs) were measured using the biosthesiometer (Bio-Medical Instrument Company, Newbury, Ohio) from both the right lateral malleolus and the right ulnar styloid process. Mean VPTs were calculated and used in the analysis. The right upper and lower limb scores from the motor and sensory tests were only included in the analysis as GABA concentration was estimated in the left sensorimotor cortex.

Magnetic Resonance Imaging Protocol

All scans were performed using a 3T Achieva system (Philips Medical Systems, Best, Netherlands) with a 32-channel head coil.

Single-Voxel Spectroscopy

Sagittal T1-, coronal T2- and axial proton density-weighted scans were used for voxel placement in the three grey matter (GM) regions (prefrontal cortex, right hippocampus and left sensorimotor cortex).

Due to the significant overlapping signals of GABA with other metabolites, non-invasive measures of GABA in the brain were acquired using the widely used MEGA-PRESS editing sequence (Mescher *et al*., 1998), with parameters TR= 2000ms, TE = 68ms, MOIST water suppression, pencil-beam automated shimming, and editing pulses centred at 1.9 and 7.5ppm on each alternate scan. The dimensions and averages for the volume of interest (VOI) were as follows: (1) right hippocampus: dimensions 30 x 19.2 x 16 mm^3, VOI volume = 9.22 mls, 576 averages; (2) prefrontal cortex: dimensions 28 x 30 x 22 mm^3, VOI volume = 18.48 mls, 432 averages; and (3) left sensorimotor cortex: dimensions 35 x 34 x 22 mm^3, VOI volume = 26.18 mls, 400 averages. The acquisition and analysis protocol used in this study followed recently published guidelines for GABA-edited MRS at 3 T using MEGA-PRESS (Mullins *et al*., 2014). Example placements of each VOI can be seen in **Figure 1**.

A non-water suppressed scan was also acquired with the same parameters (24 averages in one block), to provide an internal water reference to metabolite concentrations. TARQUIN was used to estimate the concentrations of GABA, total N-acetyl-aspartate (tNAA), and Glx (Wilson *et al*., 2011). tNAA refers to the sum of NAA (N-acetylaspartate) + NAAG (N-acetylaspartyl glutamate) and [Glx] refers to the sum of glutamate and glutamine.

Spectral Quantification

Spectral quality was assessed using Cramer-Rao-Lower-Bounds (CRLB) values provided by TARQUIN. Only data that had CRLB values of ≤20% were included in the analysis. From the participants, datasets for 6 controls and 14 patients had to be excluded from the hippocampus due to Cramer-Rao-Lower-Bounds (CRLB) values larger than 20% (Near, 2014), and one patient's dataset was excluded from the hippocampus due to time constraints. The hippocampus is technically very challenging due to its small size, the strong susceptibility to effects of nearby cranial air and bone, as well as the pulsatile cerebrospinal fluid (CSF) surrounding it (Solanky *et al.*, 2013). In the prefrontal cortex, datasets from 3 controls and 1 patient were excluded due to CRLB values greater than 20%. In the sensorimotor cortex, 2 controls and 1 patient were excluded due to CRLB values greater than 20% and 4 patient datasets were not acquired due to time constraints.

MR Imaging

Participants underwent axial proton density / T2-weighted imaging which was acquired using a dual-echo turbo spin echo (TSE) sequence (TR = 3500 ms; TE = 19/85 ms; flip angle $\alpha = 90^{o}$; FOV = 240 x 180 mm^2; with spatial resolution of 1x1x3mm^3), which was used to mark T2-hyperintense lesions. We outlined T2-hyperintense lesions in all SPMS participants on the axially acquired T2-weighted images using a semi-automated edge finding tool in JIM v. 6, then recorded the volume of T2-weighted lesions in millilitres for each subject.

For calculation of brain tissue volumes a 3D T1-weighted magnetisation-prepared gradient-echo sequence was used (TR = 6.9 ms; TE = 3.1 ms; TI = 824 ms; flip angle $\alpha = 8^{o}$; FOV = 256 x 256 mm^2; voxel size = 1 x 1 x 1 mm^3). The GM, WM and cerebrospinal fluid (CSF)

masks were obtained by segmentation of the volumetric T1-weighted scans, which were used to calculate the fractional tissue content of each spectral VOI.

Hypointense lesions on the T1-weighted volume scan were marked and filled with values consistent with normal-appearing white matter signal intensity to prevent misclassification of tissue during segmentation (Chard *et al.*, 2010). Segmentation of the lesion-filled image was then performed using the 'new_segment' function in SPM8 (statistical parametric mapping; Wellcome Trust Centre for Neuroimaging, University College London (UCL) Institute of Neurology, London). The brain parenchymal fraction (the sum of white and grey matter relative to total intracranial volume) was then recorded for each subject.

All subjects also underwent an axial 3D double inversion recovery (DIR) scan (with a spatial resolution of 1 x 1 x 3 mm^3, TR = 16000 ms; TE = 9.9 ms; T1 = 3400/325 ms). Following recent consensus recommendations, DIR lesions were marked for each patient (Geurts *et al.*, 2011). In order to count the number of T2 and DIR lesions within each spectroscopic voxel, we created a binary mask of the spectroscopic voxel by using the absolute scan geometry of the PD/T2 and DIR volumes as a reference.

Statistical analysis

Differences in cognitive and clinical performance and metabolite concentrations between groups

Differences between patients and controls were examined using independent-samples t-tests. For GABA measures, differences were examined for each region separately using multiple linear regression to adjust for age, gender, GM and WM fraction. Since the concentrations of tNAA and Glx can also be estimated within the same voxel using the same protocol, we completed the investigation by looking at these metabolites in the model.

Associations between clinical disability and regional GABA levels in patients and controls

Associations between GABA levels and clinical disability were examined in patients and controls combined using multiple regressions of the clinical variables on GABA predictors with interaction terms (i.e. group x GABA measure) to allow different associations between patients and controls to be estimated. These models allowed adjustment for covariates, such as age, gender, and premorbid IQ (when appropriate). Potential confounders, such as GM lesions within the spectroscopic VOI, anxiety and depression were also examined by including these as covariates. GM lesions within the spectroscopic VOI were not included in the final model, as they did not contribute to the model. In these models, variables describing executive function and information processing speed were regressed on prefrontal GABA concentration, those describing memory (visual and verbal) functions on hippocampus GABA concentration, and those describing right upper and lower limb motor and sensory ability on sensorimotor cortex GABA concentration.

In patients only, to assess independence of GABA concentration from tNAA or Glx as predictors, tNAA or Glx was added to models with GABA in cases where GABA was significantly or borderline significantly associated with a clinical score.

Where regression residuals showed deviation from normality, we used bias-corrected nonparametric bootstrap with 1,000 replicates to obtain confidence intervals (CIs) and p-values. Significance was set at 5% level.

All analyses were performed in Stata 13.1 (Stata Corporation, College Station, Texas, USA).

RESULTS

Participant demographics and characteristics

Thirty patients with SPMS (mean age = 51.3 yrs (SD9.6), 23 females, median EDSS=6 (range 4 – 6.5)) and 17 healthy controls (mean age = 46.3 yrs (SD11.7), 9 females) were studied. Overall patients had a short duration of progressive disease (mean duration of progressive disease = 4 yrs) and a moderate level of disability. Further details on patient demographic characteristics and disability are summarised in **Table 1.**

Clinical disability

As expected, patients had significantly slower processing speed on the SDMT ($p < 0.001$) and had worse verbal memory than controls, with significantly worse immediate ($p = 0.007$) and 30 minute delayed recall ($p = 0.017$) of the list, after adjusting for age and gender (**Table 1**). In contrast, patients and controls did not differ in their executive function (Stroop $p = 0.648$, Hayling sentence completion $p = 0.077$), working memory ($p = 0.254$), visuospatial memory ($p = 0.232$), premorbid IQ ($p = 0.092$), or on the PASAT ($p = 0.140$). 63% of patients (n = 19) were categorised as cognitively preserved, with only 37% (n = 11) categorised as cognitively impaired.

Patients performed significantly worse on all motor and sensory tests, when compared to controls, specifically, grip strength ($p = 0.001$), muscle strength ($p = 0.001$), 9-HPT ($p = 0.001$), TWT ($p = 0.001$) and VPTs ($p = 0.001$), after adjusting for age and gender (**Table 1**).

Structural MRI Measures

Total grey matter DIR lesions in patients ranged from 0 – 32, with a median of 16 lesions. The median number of grey matter lesions within each of the spectroscopic VOIs was 0 (**Table 2)**.

Patients showed significant whole brain WM atrophy when compared with controls (WM fraction: 0.323 vs. 0.340, $p < 0.0001$), after adjusting for age and gender. There was no significant difference in total brain GM fraction (GMF) ($p = 0.739$), or in the GM or WM tissue volumes within the spectroscopic voxels between patients and controls (all p values>0.05) (**Table 2**).

GABA concentration in the hippocampus and sensorimotor cortex was lower in patients than controls

Patients had significantly lower GABA concentration in the hippocampus, reduced by 0.403mM (95% CIs -0.792, -0.014, $p = 0.043$), and in the sensorimotor cortex, reduced by 0.385mM (95% CIs -0.667, -0.104, $p = 0.009$), when compared with healthy controls, after adjusting for age, gender and GMF and WMF within the spectroscopic voxel (**Table 3**). However, there was no significant difference in GABA concentration in the prefrontal cortex ($p = 0.096$) between patients and controls.

Patients also showed a significantly lower tNAA concentration in the sensorimotor cortex by 2.455mM ($p = 0.007$) when compared with healthy controls, after adjusting for age, gender and GMF and WMF within the spectroscopic voxel. There were no significant differences in tNAA levels in the hippocampus ($p = 0.512$) and prefrontal cortex ($p = 0.587$) between groups after adjusting for the above-mentioned covariates (**Table 3**).

There were no significant difference in [Glx] in the prefrontal cortex, hippocampus and sensorimotor cortex between patients and controls (**Table 3**).

Associations between GABA levels in the sensorimotor cortex and clinical scores

In patients, worse motor function in the right upper and lower limb was significantly associated with lower GABA levels in the sensorimotor cortex, after correcting for age and gender. In particular, for each unit decrease in GABA levels, there was a predicted -10.86 (95% CIs -17.786, - 4.482) decrease in grip strength (kg force) ($p < 0.001$) and -8.74 (95% CIs -13.943, -3.015) decrease in muscle strength of the right upper and lower limb ($p < 0.006$), according to the corresponding regression models. Also, per unit decrease in GABA levels, there was a predicted borderline significant decrease in the 9-HPT z-score of -1.257 (95% CIs -2.782, 0.261) ($p < 0.10$) (**Figure 2**). These significant associations did not show any substantial change (i.e., their regression coefficients did not change) when tNAA and Glx levels in the sensorimotor cortex were included into the model. Repeating the regression models including age, gender, depression, and premorbid IQ did not change the pattern of results.

There were no significant associations between GABA concentration in the sensorimotor cortex and the remaining physical disability scores, such as EDSS, TWT, and VPTs, and between GABA concentration in either the hippocampal or prefrontal VOI and any of the cognitive tests.

DISCUSSION

This study provides evidence that (1) GABA levels are reduced in the hippocampus and sensorimotor cortex in patients with SPMS and (2) lower GABA concentration in the sensorimotor cortex correlates with reduced motor function of the contralateral limbs. We will discuss each of these results in turn.

Evidence of GABAergic dysfunction in SPMS

The observed reduced GABA levels in the hippocampus and sensorimotor cortex in patients with SPMS when compared with healthy controls raises the possibility that GABA may be a marker of neurodegeneration in the brain. The reduction in GABA levels may reflect a combination of reduced GABA receptor levels and decreased density of inhibitory interneuron processes in the motor cortex in patients with progressive MS, which have been described by a previous histological study (Dutta *et al*., 2006). A PET imaging study in MS using ^{11}C-flumazenil (Freeman *et al.,* 2010), which binds the benzodiazepine site on the $GABA_A$ receptor, reported that the uptake of ^{11}C-flumazenil was lower (indicating reduced $GABA_A$ receptor levels) in the cortex of patients with multiple sclerosis (RRMS and SPMS) compared with healthy controls (Freeman *et al.,* 2010), suggesting the loss of dendrites and synapses seen at post-mortem analysis (Wegner *et al.,* 2006), which may precede the development of measurable brain atrophy. A previous study reported reduced glutamate levels in grey matter (cingulate and parietal cortices) in RRMS patients compared to controls, suggesting that a reduced availability of the precursor glutamate, may contribute to reduced synthesis of GABA. The significant decrease in tNAA concentration in the sensorimotor cortex in patients compared to controls confirms that there was significant neuronal loss

and/or dysfunction in this region, since NAA is a well-established marker of neuroaxonal integrity, viability and metabolism (Moffett *et al*., 2007).

In addition to being a marker of neurodegeneration, decreases in GABA levels may contribute to the ongoing neurodegenerative process in progressive MS. Although spectroscopic measurements in vivo do not allow us to draw firm conclusions about possible changes in the neurotransmitter pool and/or GABAergic pathway, there is some evidence from preclinical studies that MRS-derived GABA levels reflect extra-synaptic GABA tone, rather than synaptic GABA activity (Mason *et al*., 2001). Therefore, the observed reduced GABA levels may reflect a reduction in inhibitory innervation of cortical neurons, which, in turn, upregulates the firing rate of demyelinated axons, resulting in higher energy demands, as proposed by Dutta *et al*., 2006; this may ultimately result in progressive axonal loss and neurodegeneration. Additionally, there is evidence that GABA mediates neuroprotection by delaying neuronal death (Saji & Reis, 1987).

There was no difference in GABA levels in the prefrontal cortex between patients and controls, indicating a regional variation in altered GABA levels. This is likely to reflect regional variation in both the reduced synaptic density and neuronal loss, and in the possible role of altered GABA concentration, as a mechanism of plasticity and functional reorganization, as explained below.

The fact that tNAA was not a significant factor in the models including GABA, and that the RC's for GABA did not materially change when adjusting for tNAA, imply that the relationship between lower GABA and poorer clinical performance seemed not to be

confounded by the levels of tNAA, which could hypothetically be associated with GABA levels and with clinical performance.

Association between lower GABA levels and physical disability

In this study, there were associations between decreased muscle strength and worse performance on the 9-HPT, and lower sensorimotor GABA concentration. This suggests that greater loss of inhibitory neurons (and their processes) is an important contributor to clinical disability. Additionally, reduced GABA levels may represent a mechanism through which progressive axonal degeneration leads to progressive neurological disability. Patients with SPMS may have a loss of compensatory mechanisms associated with cortical reorganisation and adaptation, due to a reduction in synapses and neurons, resulting in the loss or deterioration in function.

The observed correlation between worse 9-HPT scores and lower GABA levels in the sensorimotor cortex is in contrast to a study by Bhattacharyya *et al* (2014), as previously mentioned. Bhattacharyya *et al.*, found that a worsening of performance on the 9-HPT was associated with increased GABA levels in patients with RRMS. These conflicting findings may reflect differences between patient populations (SPMS compared to RRMS). Patients with SPMS may have loss of the compensatory mechanisms associated with cortical reorganisation (Rocca *et al.,* 2005), as a result of ongoing loss of inhibitory GABA neurotransmission, ultimately resulting in progressive neurodegeneration and progressive disability. In contrast, in RRMS, reduced GABA levels are associated with improved motor performance, as a result of adaptation of cortical grey matter. The inclusion, in the Bhattacharyya's study, of patients currently taking GABAergic medications at the time of scanning is a limitation.

We found no significant associations between GABA levels in the sensorimotor cortex and VPTs, TWT or EDSS. This may be due to the role of regions other than the sensorimotor cortex in contributing to the clinical function that is captured by these scores, the small range of EDSS (4-6.5) in the patient group, and the impact of changes in regionally specific GABA levels on function.

It is surprising that patients' performance on cognitive tests were not associated with GABA levels in relevant brain regions. It is difficult to say why we did not see a relation between memory function and GABA levels in the prefrontal cortex and hippocampus grey matter regions, but this may be due to a number of reasons. First, the patients recruited into this study were relatively early in their progressive disease course (mean duration = 4 years) and had no significant GM atrophy. Second, 63% of patients (n = 19) were categorised as cognitively preserved, with only 37% (n = 11) categorised as cognitively impaired. Third, a sample size calculation with 80% power, at 5% significance, requires an N of 85 to detect a significant association between GABA levels and the cognitive tests, which is substantially more than the number recruited into this study. Finally, GABA differences may be more widespread and so have an effect in relation to memory that is not evident in the single VOI studied.

Limitations and future directions

One limitation of this study is the shape of the spectroscopic VOIs and the linear relationship between the VOI volume and spectroscopic SNR. As the regions of interest were small it was necessary to have VOIs that encompassed the GM of interest, rather than being completely contained within the specific region, in order to achieve reliable measurements of metabolite

concentrations in acceptable acquisition times. Whilst every effort was made to limit the MRS voxel to the GM, the size and shape of the VOIs meant this included some WM from surrounding tissue. The correction for WMF and GMF within the spectroscopic voxel in the statistical models will have reduced the possibility that differences in these measures between groups were responsible for differences in GABA. One study (Bhattacharyya *et al.*, 2011) measured GABA in the sensorimotor cortex with similar GMF (37+/-7%) and WMF (52+/-12%) as to those reported in **Table 2**. They found that the concentrations of GABA within the grey matter and white matter were up to nine times greater in GM compared to WM (2.87+/-0.61mM versus 0.33+/-0.11mM) (Bhattacharyya *et al.*, 2011), which suggests that the majority of the GABA concentration quantified with MRS derives from the GM.

From a technical point of view, the spectral editing cannot separate the GABA signal from the macromolecule (MM) component, which may be clinically relevant (Cudalbu *et al.*, 2012). A number of approaches have been proposed to separate GABA from co-edited MM signals, including metabolite nulling (Behar *et al.*, 1994), and symmetric editing-based suppression of MM (Henry *et al.*, 2001). Each of these methods have significant detrimental effects on the quality of the data as well as the acquisition time (Mullins *et al.*, 2014), and MM contamination is frequently accepted as a limitation of this most commonly applied approach (Mullins *et al.*, 2014). It has also been reported in the literature, that occipital cortex GABA concentration is modulated during the menstrual cycle, with reduced GABA during the follicular phase of the cycle (Epperson *et al.*, 2002). We did not correct for menstrual cycle in our analysis, but note that this variable would not be relevant as 14 out of 23 female patients and 4 of 9 female controls were menopausal.

In the future, it will be useful to follow these patients up over time to see what happens to the GABA concentration in these regions, in addition to looking at a cohort of patients with RRMS to see how GABA levels differ to patients with progressive MS. Also, it would be very useful to combine PET imaging, in particular ^{11}C-flumazenil, with ^{1}H-MRS, to investigate the co-localisation of the PET signal changes with the ^{1}H-MRS derived GABA changes.

Conclusion

Using ^{1}H-MRS, we provide the first in vivo evidence that GABA neurotransmission in the hippocampus and sensorimotor cortex is reduced in patients with SPMS when compared with healthy controls. Lower GABA levels in the sensorimotor cortex of MS patients are associated with reduced motor performance. These findings raise the possibility that altered GABA neurotransmission may be a marker of neurodegeneration, but it may also suggest that GABA is a mechanism of neurodegeneration in progressive MS patients. If we put these findings together with the evidence that GABA may mediate neuroprotection, targeting GABA may be a productive strategy that should be further explored in MS.

Figure Legend

Figure 1: Placement of MR Spectroscopy (MRS) voxels (left) with their example MRS spectra (right) in the prefrontal cortex (A), right hippocampus (B) and the left sensorimotor cortex (C) GABA - gamma-aminobutyric acid; tNAA – total N-acetyl-aspartate

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Ethics Approval University College London Hospitals NHS Ethics Committee.

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