Reduced gamma-aminobutyric acid concentration is associated with physical disability in progressive multiple sclerosis

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Neurodegeneration is thought to be the major cause of ongoing, irreversible disability in progressive stages of multiple sclerosis. Gamma-aminobutyric acid is the principle inhibitory neurotransmitter in the brain. The aims of this study were to investigate if gamma-aminobutyric acid levels (i) are abnormal in patients with secondary progressive multiple sclerosis compared with healthy controls; and (ii) correlate with physical and cognitive performance in this patient population. Thirty patients with secondary progressive multiple sclerosis and 17 healthy control subjects underwent single-voxel MEGA-PRESS (MEscher-GArwood Point RESolved Spectroscopy) magnetic resonance spectroscopy at 3 T, to quantify gamma-aminobutyric acid 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 gamma-aminobutyric acid 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 gamma-aminobutyric acid 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 gamma-aminobutyric acid levels in the hippocampus (adjusted difference = −0.403 mM, 95% confidence intervals −0.792, −0.014, \( P = 0.043 \)) and sensorimotor cortex (adjusted difference = −0.385 mM, 95% confidence intervals −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 gamma-aminobutyric acid concentration in the sensorimotor cortex. Specifically for each unit decrease in gamma-aminobutyric acid levels (in mM), there was a predicted −10.86 (95% confidence intervals −16.786 to −4.482) decrease in grip strength (kg force) (\( P < 0.001 \)) and −8.74 (95% confidence intervals −13.943 to −3.015) decrease in muscle strength (\( P < 0.006 \)). This study suggests that reduced gamma-aminobutyric acid levels reflect pathological abnormalities that may play a role in determining physical disability. These abnormalities may include decreases in the pre- and postsynaptic components of gamma-aminobutyric acid neurotransmission and in the density of inhibitory neurons. Additionally, the reduced gamma-aminobutyric acid concentration may contribute to the neurodegenerative process, resulting 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 gamma-aminobutyric acid neurotransmission may be an important target for neuroprotection in multiple sclerosis.

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Introduction

There is a need to understand the mechanisms of neurodegeneration in progressive multiple sclerosis (Fox et al., 2012). Secondary progressive multiple sclerosis develops after an initial relapsing remitting multiple sclerosis 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). Secondary progressive multiple sclerosis 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), affecting information processing speed (Amato et al., 2010a, b), episodic memory and executive function (Strober et al., 2009). The pathological processes underlying clinical disability in multiple sclerosis 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 patients with multiple sclerosis, reduced concentrations of grey matter glutamate, the main excitatory neurotransmitter of the human brain, correlated with worse memory function (Muhlert et al., 2014), suggesting that abnormalities in the neurotransmitter pathways may play a role in neurodegeneration, which underpins disability in multiple sclerosis.

Gamma-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the brain (DeFelipe, 1993) and is produced from glutamate by l-glutamic acid decarboxylase within GABAergic neurons (Chang et al., 2003). GABA is then metabolized to succinic acid semialdehyde by GABA transaminase and then to succinate, mainly within astrocytic mitochondria (Chang et al., 2003). GABA is needed for normal brain function, synaptic plasticity, cortical adaptation and reorganization (Stagg, 2014). Altered GABA concentrations, either increases or decreases, have been detected in a number of conditions, including epilepsy (MacDonald et al., 2010), and schizophrenia (Reynolds et al., 2002). GABAergic inhibition may be one of the mechanisms involved in use-dependent plasticity in the intact human motor cortex (Levy et al., 2010). Changes in GABA concentration in the sensorimotor cortex during motor learning have been demonstrated (Floyer-Lea et al., 2006). One pilot study in relapsing-remitting multiple sclerosis (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 reorganization occurring in the sensorimotor cortex in patients with relapsing-remitting multiple sclerosis, as reflected by increased functional MRI 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 1H-magnetic resonance spectroscopy is challenging due to the spectral overlap of GABA with more abundant metabolites, such as NAA at 2 ppm (Puts and Edden, 2012). MEGA-PRESS (MEscher-GArwood Point REsolved Spectroscopy) is a spectral editing method that allows the discrimination of GABA from these metabolites (Mescher et al., 1998); it also allows quantification of total N-acetyl-aspartate (NAA), which, when reduced, indicates neuronal loss and/or metabolic dysfunction (Moffett et al., 2007), and the glutamine–glutamate complex (Glx), which has been found to be reduced in the spinal cord of patients with primary progressive multiple sclerosis compared to healthy controls, suggesting 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 magnetic resonance spectroscopy studies in progressive multiple sclerosis (Sastre-Garriga et al., 2005); (ii) reduced GABA-related gene transcripts and density of inhibitory neuronal processes in the motor cortex of autopsied multiple sclerosis brains (Dutta et al., 2006); and (iii) impaired compensatory mechanisms occurring in secondary progressive multiple sclerosis compared with relapsing-remitting multiple sclerosis (Rocca et al., 2005), lead us to hypothesize that GABA levels are reduced in patients with secondary progressive multiple sclerosis when compared with healthy controls and that they correlate with increased clinical disability. 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 magnetic resonance spectroscopy. 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 total NAA and Glx, obtained within the same spectroscopic voxels.

Materials and methods

Subjects

Patients with a diagnosis of secondary progressive multiple sclerosis 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 this cross-sectional standalone study. Healthy control subjects 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 Shellice, 1997). 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 the Hospital Anxiety and Depression Scale (Zigmond and Smith, 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 standard deviations (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 EDSS (Kurtzke, 1983). All patients and controls were also assessed using the 9-Hole Peg Test (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-Hole Peg Test 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 Incorp.) (Svens and Lee, 2005). The average of two trials for the TWT and the average of two trials for the 9-Hole Peg Test were calculated (Fischer et al., 1999). Vibration perception thresholds were measured using the biothesiometer (Bio-Medical Instrument Company) from both the right lateral malleolus and the right ulnar styloid process. Mean vibration perception thresholds 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.

MRI protocol

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

Structural MRI

All participants underwent structural imaging, which included: (i) axial proton density/T2-weighted imaging using a 2D dual-echo turbo spin echo (TSE) sequence (repetition time = 3500 ms; echo time = 19/85 ms; flip angle α = 90°; field of view = 240 × 180 mm²; with spatial resolution of 1 × 1 × 3 mm³); (ii) 3D MPRAGE (T₁-weighted magnetization-prepared rapid acquisition gradient-echo sequence) (repetition time = 6.9 ms; echo time = 3.1 ms; inversion time = 824 ms; flip angle α = 8°; field of view = 256 × 256 mm²; voxel size = 1 × 1 × 1 mm³); and (iii) axial 2D DIR (double inversion recovery) scan (voxel-size = 1 × 1 × 3 mm³, repetition time = 16 000 ms; echo time = 9.9 ms; inversion time = 3400/325 ms).

We outlined hyperintense lesions in all secondary progressive multiple sclerosis 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.
Hypointense lesions on the T₂-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 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. For calculation of brain tissue volumes the grey matter, white matter and CSF masks were obtained by segmentation of the volumetric T₁-weighted scans.

Double inversion recovery lesions were marked for each patient following consensus recommendations (Geurts et al., 2011).

**Single-voxel spectroscopy**

Sagittal T₁-, coronal T₂- and axial proton density-weighted scans were used for voxel placement in the three grey matter 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 repetition time = 2000 ms, echo time = 68 ms, MOIST water suppression, pencil-beam automated shimming, and editing pulses centred at 1.9 and 7.5 ppm on each alternate scan. The dimensions and averages for the volume of interest were as follows: (i) right hippocampus: dimensions 30 × 19.2 × 16 mm³, volume = 9.22 ml, 576 averages; (ii) prefrontal cortex: dimensions 28 × 30 × 22 mm³, volume = 18.48 ml, 432 averages; and (iii) left sensorimotor cortex: dimensions 35 × 34 × 22 mm³, volume = 26.18 ml, 400 averages. The acquisition and analysis protocol used in this study followed recently published guidelines for GABA-edited magnetic resonance spectroscopy at 3 T using MEGA-PRESS (Pullins et al., 2014). Example placements of each volume of interest can be seen in Fig. 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 NAA, and Glx (Wilson et al., 2011). Total NAA refers to the sum of NAA + NAAG (N-acetylaspartyl glutamate) and Glx refers to the sum of glutamate and glutamine.

**Spectral quantification**

Spectral quantification was carried out using TARQUIN (Totally Automatic Robust Quantification in NMR), which provides a fully automatic analysis of in vivo spectra (Wilson et al., 2011). The analysis package uses a linear combination of basis functions to fit spectra, with analysis performed in the time domain (Wilson et al., 2011). For MEGA-PRESS data, TARQUIN uses a simple predefined basis set which models the GABA peak as two single Gaussian peaks (Pullins et al., 2012). The reliability of TARQUIN has been demonstrated to be comparable to other spectral quantification methods (O’Gorman et al., 2011). The non-water suppressed scan was used as an internal reference of known concentration to find the absolute concentrations of GABA.

Spectral quality was assessed using Cramér-Rao-Lower-Bounds (Rao, 1946) values provided by TARQUIN, which represent the minimum possible variance on a fit parameter. Only data that had Cramér-Rao-Lower-Bounds values of ≤20% were included in the analysis. From the participants, data sets for six controls and 14 patients had to be excluded from the hippocampus due to Cramér-Rao-Lower-Bounds values >20% (Nar, 2014), and one patient’s data set 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 CSF surrounding it (Solanky et al., 2013). In the prefrontal cortex, data sets from three controls and one patient were excluded due to Cramér-Rao Lower Bounds values >20%. In the sensorimotor cortex, two control subjects and one patient were excluded due to Cramér-Rao Lower Bounds values >20% and four patient data sets were not acquired due to time constraints.

The fractional content of grey matter and white matter within each spectroscopic voxel was calculated. To count the number of T₂ and double inversion recovery lesions within each spectroscopic voxel, we created a binary mask of the spectroscopic voxel by using the absolute scan geometry of the proton density/T₂ and double inversion recovery 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, grey matter and white matter fraction. As the concentrations of total NAA 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 × 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 grey matter lesions within the spectroscopic volume of interest, anxiety and depression were also examined by including these as covariates. Grey matter lesions within the spectroscopic volume of interest 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 total NAA or Glx as predictors, total NAA 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 non-parametric bootstrap with 1000 replicates to obtain confidence intervals (CIs) and

![Figure 1 Examples of the MR spectra. Placement of magnetic resonance spectroscopy voxels (left) with their example magnetic resonance spectroscopy spectra (right) in the prefrontal cortex (A), right hippocampus (B) and the left sensorimotor cortex (C). tNAA = total NAA.](image-url)
Results

Participant demographics and characteristics

Thirty patients with secondary progressive multiple sclerosis [mean age = 51.3 years (SD 9.6), 23 females, median EDSS = 6 (range 4–6.5)] and 17 healthy control subjects [mean age = 46.3 years (SD 11.7), nine females] were studied. Overall patients had a short duration of progressive disease (mean duration of progressive disease = 4 years) and a moderate level of disability. Further details on patient demographic characteristics and disability are summarized 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 min 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). Sixty-three per cent of patients (n = 19) were categorized as cognitively preserved, with only 37% (n = 11) categorized as cognitively impaired.

Patients performed significantly worse on all motor and sensory tests, when compared to control subjects, specifically grip strength (P = 0.001), muscle strength (P = 0.001), 9-Hole Peg Test (P = 0.001), TWT (P = 0.001) and vibration perception thresholds (P = 0.001), after adjusting for age and gender (Table 1).

Structural MRI measures

Total grey matter double inversion recovery 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 volumes of interest was 0 (Table 2).

Patients showed significant whole brain white matter atrophy when compared with control subjects (white matter fraction: 0.323 versus 0.340, after adjusting for age and gender. There was no significant difference in total brain grey matter fraction (P = 0.739), or in the grey matter or white matter 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 0.403 mM (95% CIs –0.792, –0.014, P = 0.043), and in the sensorimotor cortex,
Table 2 MRI measures in patients and controls

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Patients</th>
<th>Adjusted difference* (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total brain GMF</td>
<td>0.4719 (0.015)</td>
<td>0.4718 (0.006)</td>
<td>0.001 (−0.005 to 0.007)</td>
<td>0.739</td>
</tr>
<tr>
<td>Total brain WMF</td>
<td>0.340 (0.014)</td>
<td>0.323 (0.016)</td>
<td>−0.018 (−0.027 to −0.009)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Brain parenchymal fraction</td>
<td>0.812 (0.020)</td>
<td>0.796 (0.019)</td>
<td>−0.015 (−0.025 to −0.004)</td>
<td>0.008</td>
</tr>
<tr>
<td>GMF in the hippocampus</td>
<td>0.540 (0.096)</td>
<td>0.582 (0.085)</td>
<td>0.046 (−0.027 to 0.119)</td>
<td>0.209</td>
</tr>
<tr>
<td>WMF in the hippocampus spectroscopic VOI</td>
<td>0.442 (0.086)</td>
<td>0.384 (0.079)</td>
<td>−0.062 (−0.130 to 0.005)</td>
<td>0.070</td>
</tr>
<tr>
<td>GMF in the prefrontal cortex spectroscopic VOI</td>
<td>0.496 (0.059)</td>
<td>0.499 (0.034)</td>
<td>0.017 (−0.012 to 0.045)</td>
<td>0.242</td>
</tr>
<tr>
<td>WMF in the prefrontal cortex spectroscopic VOI</td>
<td>0.348 (0.069)</td>
<td>0.334 (0.081)</td>
<td>−0.026 (−0.080 to 0.028)</td>
<td>0.329</td>
</tr>
<tr>
<td>GMF in the sensorimotor cortex spectroscopic VOI</td>
<td>0.323 (0.036)</td>
<td>0.350 (0.095)</td>
<td>0.031 (−0.024 to 0.087)</td>
<td>0.261</td>
</tr>
<tr>
<td>WMF in the sensorimotor cortex spectroscopic VOI</td>
<td>0.575 (0.069)</td>
<td>0.533 (0.135)</td>
<td>−0.052 (−0.129, 0.026)</td>
<td>0.184</td>
</tr>
<tr>
<td>DIR lesions (median) (range)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total brain grey matter lesions</td>
<td>NA</td>
<td>16 (0–32)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Grey matter lesions in the prefrontal spectroscopic VOI</td>
<td>NA</td>
<td>0 lesions (range 0–4)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Grey matter lesions in the hippocampal spectroscopic VOI</td>
<td>NA</td>
<td>0 lesions (range 0–1)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Grey matter lesions in the sensorimotor cortex spectroscopic VOI</td>
<td>NA</td>
<td>0 lesions (range 0–2)</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

**DIR** = double inversion recovery; **GMF** = grey matter fraction; **WMF** = white matter fraction; **VOI** = spectroscopic volume of interest.

*Adjusted for age and gender.

Reduced by 0.385 mM (95% CIs −0.667, −0.104, P = 0.009), when compared with healthy controls, after adjusting for age, gender and grey matter and white matter fractions 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 total NAA concentration in the sensorimotor cortex by 2.455 mM (P = 0.007) when compared with healthy controls, after adjusting for age, gender and grey matter and white matter fractions within the spectroscopic voxel. There were no significant differences in total NAA levels in the hippocampus (P = 0.512) and prefrontal cortex (P = 0.587) between groups after adjusting for the abovementioned 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 −10.861 (95% CIs −17.786, −4.482) decrease in grip strength (kg force) (P < 0.001) and −8.736 (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-Hole Peg Test z-score of −1.257 (95% CIs −2.782, 0.261) (P < 0.10). These significant associations did not show any substantial change (i.e. their regression coefficients did not change) when total NAA 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 vibration perception thresholds, and between GABA concentration in either the hippocampal or prefrontal volumes of interest and any of the cognitive tests.

**Associations between total NAA levels in the sensorimotor cortex and clinical scores**

In patients, lower total NAA in the sensorimotor cortex was also significantly associated with worse motor function, after correcting for age and gender. For each unit decrease in total NAA levels, there was a predicted −1.20 (95% CIs −0.475, −1.98) decrease in grip strength (P < 0.015), −0.943 (95% CIs −0.734, −1.828) decrease in muscle strength (P < 0.045) and −0.142 (95% CIs
Discussion

This study provides evidence that (i) GABA levels are reduced in the hippocampus and sensorimotor cortex in patients with secondary progressive multiple sclerosis; and (ii) 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 secondary progressive multiple sclerosis

The observed reduced GABA levels in the hippocampus and sensorimotor cortex in patients with secondary progressive multiple sclerosis 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 multiple sclerosis, which have been described by a previous histological study (Dutta et al., 2006). A PET imaging study in multiple sclerosis using 11C-flumazenil (Freeman et al., 2010), which binds the benzodiazepine site on the GABA_A receptor, reported that the uptake of 11C-flumazenil was lower (indicating reduced GABA_A receptor levels) in the cortex of patients with multiple sclerosis (relapsing-remitting and secondary progressive multiple sclerosis) 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 relapsing-remitting patients compared to controls, suggesting that a reduced availability of the precursor glutamate, may contribute to reduced synthesis of GABA. The significant decrease in total NAA concentration in the sensorimotor cortex in patients compared to controls confirms that there was significant neuronal loss and/or dysfunction in this region, because 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 multiple sclerosis. 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 magnetic resonance spectroscopy-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 and 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.

Association between lower GABA levels and physical disability

In this study, there were associations between decreased muscle strength and worse performance on the 9-Hole Peg Test, 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 secondary progressive multiple sclerosis may have a loss of compensatory mechanisms associated with cortical reorganization and adaptation, due to a reduction in synapses and neurons, resulting in the loss or deterioration in function.

The observed correlation between worse 9-Hole Peg Test scores and lower GABA levels in the sensorimotor cortex is in contrast to a study by Bhattacharyya et al. (2013), as previously mentioned. Bhattacharyya et al. (2013) found that a worsening of performance on the 9-Hole Peg Test was associated with increased GABA levels in patients with relapsing-remitting multiple sclerosis. These conflicting findings may reflect differences between patient populations (secondary progressive multiple sclerosis compared to relapsing-remitting multiple sclerosis). Patients with secondary progressive multiple sclerosis may have loss of the compensatory mechanisms associated with cortical reorganization (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 relapsing remitting multiple sclerosis, reduced GABA levels are associated with improved motor performance, as a result of adaptation of cortical grey matter. The inclusion, in the Bhattacharyya et al. (2013) 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 vibration perception thresholds, 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 grey matter atrophy. Second, 63% of patients (n = 19) were categorized as cognitively preserved, with only 37% (n = 11) categorized 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 volume of interest studied.

Association between lower total NAA levels and physical disability

The observed reduction in total NAA in the sensorimotor cortex in patients compared to controls is in keeping with previous studies of reduced total NAA in multiple sclerosis (Achnichts et al., 2013; Kirov et al., 2013). Total NAA is a well-established marker of neuronal loss and/or metabolic dysfunction (Moffett et al., 2007). Total NAA correlated with grip strength, muscle strength and the 9-Hole Peg Test. Several studies in multiple sclerosis have demonstrated consistent correlations with total NAA and physical disability (De Stefano et al., 1997; Aboul-Enein et al., 2010). These studies confirm neuroaxonal damage as a mechanism of disability in multiple sclerosis. Nevertheless, the fact that total NAA was not a significant factor in the models including GABA, and that the regression coefficients for GABA did not materially change when adjusting for total NAA, indicates that the relationship between lower GABA and poorer clinical performance is confirmed and independent of the levels of total NAA.

Limitations and future directions

One limitation of this study is the shape of the spectroscopic volumes of interest and the linear relationship between the volume of interest and spectroscopic signal-to-noise ratio. As the regions of interest were small, it was necessary to use volumes of interest that encompassed the grey matter of interest, rather than being completely contained within the specific region, in order to achieve reliable measurements of metabolite concentrations in acceptable acquisition times. While every effort was made to limit the magnetic resonance spectroscopy voxel to hippocampal grey matter, the size and shape of the volumes of...
interest meant this included some white matter from surrounding tissue, and small parts of neighbouring medial temporal lobe structures. This was necessary in order to attain a sufficient signal-to-noise ratio, in an acceptable acquisition time. The correction for white matter fraction and grey matter fraction 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 grey matter fraction (37 ± 7%) and white matter fraction (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 grey matter compared to white matter (2.87 ± 0.61 mM versus 0.33 ± 0.11 mM) (Bhattacharyya et al., 2011), which suggests that the majority of the GABA concentration quantified with magnetic resonance spectroscopy derives from the grey matter.

From a technical point of view, the spectral editing cannot separate the GABA signal from the macromolecule component, which may be clinically relevant (Cudalbu et al., 2012). A number of approaches have been proposed to separate GABA from co-edited macromolecule signals, including metabolite nulling (Behar et al., 1994), and symmetric editing-based suppression of macromolecules (Henry et al., 2001). Each of these methods has significant detrimental effects on the quality of the data as well as the acquisition time (Mullins et al., 2014), and macromolecule 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 of 23 female patients and four menstrual cycle (Epperson et al., 2001). Reduced NAA-levels in the NAWM of patients with MS is a feature of progression. A study with quantitative magnetic resonance spectroscopy at 3 Tesla. PLoS One 2010; 5: e11625.

Conclusion

Using 1H-magnetic resonance spectroscopy, we provide the first in vivo evidence that GABA neurotransmission in the hippocampus and sensorimotor cortex is reduced in patients with secondary progressive multiple sclerosis when compared with healthy controls. Lower GABA levels in the sensorimotor cortex of multiple sclerosis 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 multiple sclerosis 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 multiple sclerosis.

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