Journal of the Neurological Sciences 407 (2019) 116458

Contents lists available at ScienceDirect

Journal of the Neurological Sciences

journal homepage: www.elsevier.com/locate/jns

Altered hypothalamic metabolism in early multiple sclerosis – MR spectroscopy study

Petra Hnilicová[a,*], Ema Kantorová[b], Hubert Poláček[c], Marián Grendár[d], Michal Bittšanský[a], Daniel Čierny[e], Štefan Sivák[b], Kamil Zeleňák[f], Ján Lehotský[g], Dušan Dobrota[g], Egon Kurča[b]

[a] Biomedical Center Martin - Division of Neurosciences, Jessenius Faculty of Medicine in Martin, Comenius University in Bratislava, Martin, Slovakia
[b] Clinic of Neurology, Jessenius Faculty of Medicine in Martin, Comenius University in Bratislava, Martin, Slovakia
[c] Clinic of Nuclear Medicine, Jessenius Faculty of Medicine in Martin, Comenius University in Bratislava, Martin, Slovakia
[d] Biomedical Center Martin - Bioinformatic unit, Jessenius Faculty of Medicine in Martin, Comenius University in Bratislava, Martin, Slovakia
[e] Department of Clinical Biochemistry, Jessenius Faculty of Medicine in Martin, Comenius University in Bratislava, Martin, Slovakia
[f] Clinic of Radiology, Jessenius Faculty of Medicine in Martin, Comenius University in Bratislava, Martin, Slovakia
[g] Department of Medical Biochemistry, Jessenius Faculty of Medicine in Martin, Comenius University in Bratislava, Martin, Slovakia

ARTICLE INFO

Keywords:
Multiple sclerosis
Hypothalamus
^{1}H MRS
Glutamate excitotoxicity

ABSTRACT

Multiple sclerosis (MS) is a disease characterized by overlapping processes of neuroinflammation and neuroaxonal degeneration. Disturbances of the hypothalamo-pituitary axis in MS are supposed to modulate neuroinflammatory circuits, however, there is insufficient knowledge about the hypothalamic metabolism alterations in early MS. This ^{1}H MRS study performed on a 1.5 T MR-scanner was focused on the hypothalamus of 31 pre-treatment patients after their first clinical MS episode/s, compared to 31 healthy controls. The metabolite ratios of *N*-acetyl-aspartate & *N*-acetyl-aspartyl-glutamate (tNAA), glutamate & glutamine (Glx), myo-Inositol (mIns), choline- and creatine-containing compounds (tCho, tCr) were further correlated with the Expanded Disability Status Scale (EDSS). In the hypothalamus of early MS patients compared to controls, we found decreased tNAA/tCr and increased tCho/tNAA, mIns/tNAA, Glx/tCr, and Glx/tNAA. In addition, tCho/tNAA, Glx/tNAA, and mIns/tNAA were positively and tNAA/tCr was negatively correlated with EDSS. Results suggest that the decline of the tNAA ratio, indicating neuro-axonal dysfunction in the hypothalamus, may be linked with glutamate excitotoxicity. Excessive glutamate concentrations may cause microglial activation and myelinated tracts degradation with subsequent gliosis, paralleled by increased mIns and tCho ratios. This indicates that glutamate excitotoxicity can play an important role in MS from its earliest stages.

1. Introduction

Multiple sclerosis (MS) is traditionally characterized as a predominantly inflammatory disease of the central nervous system (CNS), which initiates myelin loss and axonal as well as neuronal damage [1–3]. Despite the widely accepted concomitant neurodegeneration in MS, the mechanisms of its initiation and further development are not yet sufficiently understood [1,4,5]. The deep grey matter (DGM) brain structures had been found altered since the early stages of MS [1,6] and their damage may determine the MS progression, physical disability, or cognitive impairment [1,7,8]. In this case-control study, we evaluated the metabolism of the hypothalamus due to its wide afferentation and central position in the hypothalamic-pituitary axis, which influences many autonomic, endocrine, and limbic functions altered in MS [9–11]. Although the hypothalamus is one of the most complex and essential DGM regions, it is often overlooked when investigating both the etiology and treatment of MS [4,5,10]. Based on our previous research of relapsing-remitting (RRMS) and secondary progressive (SPMS) patients [11,12], we hypothesize that the extent of hypothalamus damage may determine the severity and course of MS even in the early, pre-treatment stages of the disease. Using proton magnetic resonance spectroscopy (^{1}H MRS) we evaluated common metabolites that potentially reflect inflammatory and neurodegenerative changes in the hypothalamus. Based on recent research including our findings, we hypothesized that neurodegenerative processes in early MS stages could be initiated by an increased accumulation of extracellular glutamate (the main part of glutamate and glutamine complex; Glx). Although glutamate is the main excitatory neurotransmitter, playing an

* Corresponding author at: Biomedical Center Martin - Division of Neurosciences, Jessenius Faculty of Medicine in Martin, Comenius University in Bratislava, Mala hora 11161/4D, 036 01, Martin, Slovakia.
E-mail address: petra.hnilicova@jfmed.uniba.sk (P. Hnilicová).

https://doi.org/10.1016/j.jns.2019.116458
Received 10 January 2019; Received in revised form 19 August 2019; Accepted 10 September 2019
Available online 11 September 2019
0022-510X/

important role in the development and neuroplasticity of CNS [3,9,13,14], it might contribute to the neuro-axonal damage in MS during oxidative and/or metabolic stress [13–16]. The dysfunction of neurones and their connections, observed in MS lesions, normal appearing white matter (NAWM) as well as in cortical GM, is primarily associated with the decline of *N*-acetyl aspartate (predominant compartment of *N*-acetyl aspartate and *N*-acetyl-aspartyl-glutamate complex; tNAA) [17–19]. This almost exclusively neuronal amino acid can be a reservoir of Glx [20–22]. Excessive glutamate concentrations may also affect neuroglial homeostasis and cause microglial activation and subsequent gliosis [20,23], which is associated with increased myo-Inositol (mIns), a marker of glial cellularity [21,24,25]. Gliosis has already been confirmed in inflammatory MS lesions as well as in NAWM [16,21,25] and may precede tNAA reduction in MS [26,27]. Furthermore, mIns is supposed to be a precursor of phospholipid membrane constituents important for the formation and/or breakdown of myelin [20,21,23]. Signals from choline-containing compounds (i.e. phosphatidylcholine, glycerophosphatidylcholine, acetylcholine, and choline; tCho) [20,21,28] reflect the cell-membrane metabolism and increased level of cell-membrane turnover as seen in demyelination, remyelination, inflammation and ongoing gliosis in MS patients [18,21,25,28]. According to the traditional inflammatory theory of MS development, alterations in tCho and mIns [6,19,29] should dominate in early MS stages, the potential role of glutamate and tNAA changes can be guessed from previous work [11,12], but has not been targeted.

2. Methods

All procedures involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Ethics approval was performed by the Ethic Committee of the Jessenius Faculty of Medicine in Martin, Comenius University in Bratislava (EK 1678/2015).

2.1. Study participants

Thirty-one adult patients with an early stage of MS and thirty-one age- and gender- matched healthy volunteers were included in this study after institutional review board approval and obtaining written, informed consent. Thirteen of the MS patients were defined as having clinical isolated syndrome (CIS) and eighteen as clinical definite MS (CDMS) according to the McDonald criteria [30]. Of the 31 MS patients studied, 13 presented with optic neuritis, 9 with brainstem, 6 with hemisphere, and 3 with spinal cord syndromes. Furthermore, all patients were classified according to symptomatic monofocal (9 patients) or multifocal (22 patients) clinical MS presentation. Regarding progression to the next diseases, all MS patients had a relapsing-remitting MS phenotype. At the time of ^{1}H MRS examination, they had not been treated with any immunomodulatory agents or antidepressants. In this study, the interval between the first clinical symptoms and the date of MRS examination was defined as the disease duration. Clinical disability was evaluated by neurologists specialized in MS using the Expanded Disability Status Scale (EDSS). Structural MR images of all subjects were inspected for macroscopic WM lesions by a radiologist blinded to the participant's disease status. None of the healthy subjects demonstrated pathological findings on MRI, had any known history of endocrine, oncological, neurological or other serious disorders. Basic clinical information about patients and healthy controls is included in Table 1.

2.2. Measurement protocol

All investigations were performed on a 1.5 T whole body MR scanner Magnetom Symphony (Siemens, Erlangen, Germany) with 30 mT/m total gradient strength and 125 T/m/s nominal slew. The body coil was used for transmission and the 8-channel head coil (Siemens, Erlangen, Germany) was used for signal reception. Each study subject underwent structural MRI scanning included T_2-weighted and fluid attenuation inversion recovery (FLAIR) sequences before spectroscopy measurement. To ensure a similar volume of interest (VOI) placement between subjects, T_1-weighted magnetization prepared rapid gradient echo (MP-RAGE) anatomical reference images were obtained and subsequently resliced in three orthogonal directions. The hypothalamus is a challenging brain area for ^{1}H MRS acquisition due to its small size, central location, and air-tissue interface, especially in marginal brain regions located close to sinuses [28,31]. Furthermore, the larger voxel selection in the hypothalamic region is impaired by magnetic field inhomogeneity, as well as by encompassing larger cerebro-spinal fluid (CSF) volumes, especially in atrophic tissue, which could account for the less reliable measurements [21,32,33]. To avoid these limitations, in this study single-voxel ^{1}H-MRS was applied, rather than multi-voxel, due to its advantages of a smaller voxel size, shorter measurement time for duplicate (metabolite, water) acquisition, and the possibility of voxel shifting during post-processing [2,11,17]. To identify magnetic field (B_0) inhomogeneity, B_0 phase maps were obtained, on that shimming volumes were manually localized outside of visible B_0 distortions. Maximum possible B_0 homogeneity was then achieved by suppression slabs positioned on a magnetically heterogeneous area, and by iterative field mapping with the calculation of appropriate shim currents of the first- and second-order corrections. Three-dimensional spectroscopic imaging sequence (chemical shift imaging; CSI) based on point resolved spectroscopy (PRESS), with an echo time of TE = 30 ms, repetition time TR = 1500 ms, and 4 averages, was used to acquire localized data focused on the hypothalamic region with VOI = 50 × 60 × 35 mm^3, field of view (FOV) = 100 × 100 × 100 mm^3 and 10 × 10 × 8 acquired matrix size (i.e. 10 × 10 × 12.5 mm^3 nominal voxel size) interpolated to a 16 × 16 × 8 CSI matrix. During the ^{1}H MRS data acquisition (7 min 42 s acquisition time), the signal from the magnetically heterogeneous area was suppressed by suppression slabs (Fig. 1). The total measuring time of the whole MR examination was about 25 min = 4 min 6 s (localizer) + 8 min 51 s (MP-RAGE) + 1 min 57 s (T_2-weighted MRI) + 1 min 49 s (FLAIR) + 32 s (B_0 map) + 7 min 42 s (^{1}H MRS).

2.3. Data processing

The anatomical MRI of all subjects were inspected for macroscopic brain lesions. Based on the overlapping of MP-RAGEs with the spectroscopic CSI matrix in the graphical user interface jSIPRO (Version 1.0; Filip Jiru & Antonin Skoch, IKEM, Prague, Czech Republic), it was possible to shift the CSI grid and precisely localize voxels in the right and left hypothalamus. The voxel center was placed between the anterior commissure and the mammillary body, 1 mm anteriorly to fornix in sagittal and 2 mm laterally to the border of the 3rd ventricle in the trans-axial plane (Fig. 2). The spectra of the required two voxels were evaluated by the automatic data processing software LCModel (Version 6.2-1 L; Stephen Provencher, LCModel, Oakville, ON, Canada). After the integrals of metabolite were evaluated, the metabolite ratios to tNAA and creatine containing compounds (mainly creatine and phosphocreatine, tCr) were obtained: tNAA/tCr, tCho/tNAA, tCho/tCr, Glx/tCr, Glx/tNAA, mIns/tCr, and mIns/tNAA. All MR spectra fulfilled defined spectral quality criteria, i.e. spectral signal-to-noise-ratio (SNR) values were in all cases higher than 5 (SNR in average 9 ± 3 standard deviation). The tNAA peak amplitudes showed symmetry with the width-at-half-maximum (FWHM) lower than 10 Hz (FWHM in average 6 Hz ± 2 standard deviation). LCModel manifested proper residual signals and no spectral artefacts.

2.4. Statistical evaluation

Since the mean values of all metabolite ratios from both

Table 1
Basic clinical information about study participants.

	MS patients	Controls	*p*-value
Number	31	31	1 Two sample t-test
Age (years, mean ± SD)	27 ± 6	27 ± 6	1 Test of the equality of population proportions in two samples
Gender (M/F)	12/19	12/19	1/1 Two sample t-test
Race / ethnicity / country of origin	white/European/Slovakia		–
Diseases duration (months, mean ± SD)	5 ± 2	NA	–
EDSS median (IQR)	2.0 (2.0)	NA	–
WM lesion load (≤20 vs. > 20 lesions)	17/14	NA	–

The table shows basic clinical information about patients with early MS stages (MS patients) and healthy controls. Abbreviations: M = male, F = female, EDSS = Expanded Disability Status Scale, NA = not applicable, SD = standard deviation, IQR = interquartile range.

hypothalamic voxels were gender-independent (one-way ANOVA test) in early MS patients as well as in controls and no statistical trend to side difference was found (one-way ANOVA test), all hypothalamic metabolite values (i.e. mean values of left and right side spectra of both genders together) were used for statistical analyses. Spectroscopic differences in early MS patients vs. healthy controls, CIS vs. CDMS patient subgroups, low- vs. high-lesion load (≤20 vs. > 20 lesions), short- vs. long-disease duration (≤6 vs. > 6 months), mono- vs. multifocal clinical onset, and optic neuritis vs. other brain area degeneration were evaluated using 2-tailed 2-sample *t*-test for normally distributed data and Wilcoxon signed-rank test for data that cannot be assumed to be normally distributed (Kolmogorov-*Smirnov test; KS)*. Associations between metabolite ratios and EDSS (normally distributed according to KS *test)* were further assessed using Pearson correlation analysis. To clarify statistical differences in correlations of metabolite and EDSS referencing to tCr or tNAA, Fisher's r-to-z transformation was used. The significance level was determined at 0.05. All statistical procedures were performed using the SPSS software package (Version 15.0, Chicago, IL, USA).

3. Results

Among the patients' group, in selected metabolite ratios from both hypothalamic voxels, differences dependent on clinical MS subgroups, WM lesion load, disease duration, and clinical onset were not revealed. Also, the results of ^{1}H MRS differences between the patients with optic neuritis and patients with other brain area neurodegeneration did not confirm significant differences in metabolite ratios, except of both mIns ratios (Table 2).

The analysis of alterations in hypothalamic metabolism in the whole group of patients with early MS stages revealed significantly decreased tNAA/tCr and increased tCho/tNAA, mIns/tNAA, Glx/tCr, and Glx/tNAA, compared to controls. No group differences, neither in tCho/tCr nor mIns/tCr, were observed (Table 3).

In the hypothalamus of early MS patients, all examined metabolite ratios were positively correlated to the EDSS, except that of tNAA/tCr in which the Pearson correlation was inverse (Fig. 3).

Furthermore, the analysis of Pearson correlations between EDSS and metabolite ratios in MS patients revealed the significance of the linear

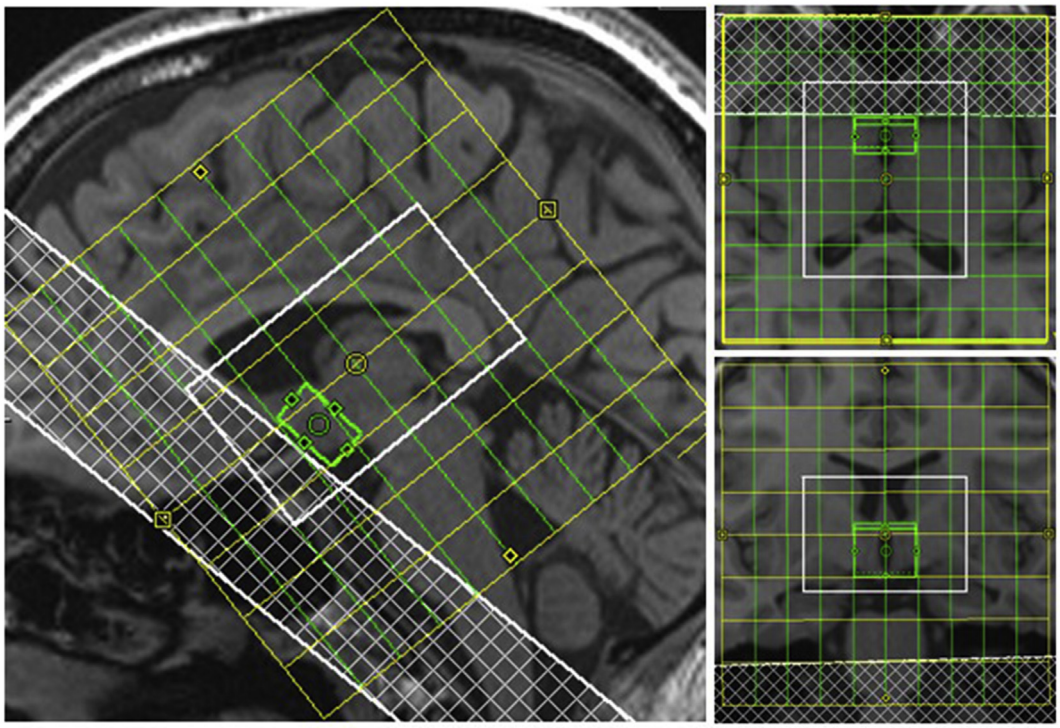

Fig. 1. Positioning of ^{1}H MRS in hypothalamus.
Anatomical MP-RAGEs display the positioning of VOI (50 × 60 × 35 mm^3), FOV (100 × 100 × 100 mm^3), and shimming volume (green rectangle) placed in the hypothalamic region. The acquired CSI matrix size was 10 × 10 × 8, with a nominal voxel size of 10 × 10 × 12.5 mm^3.

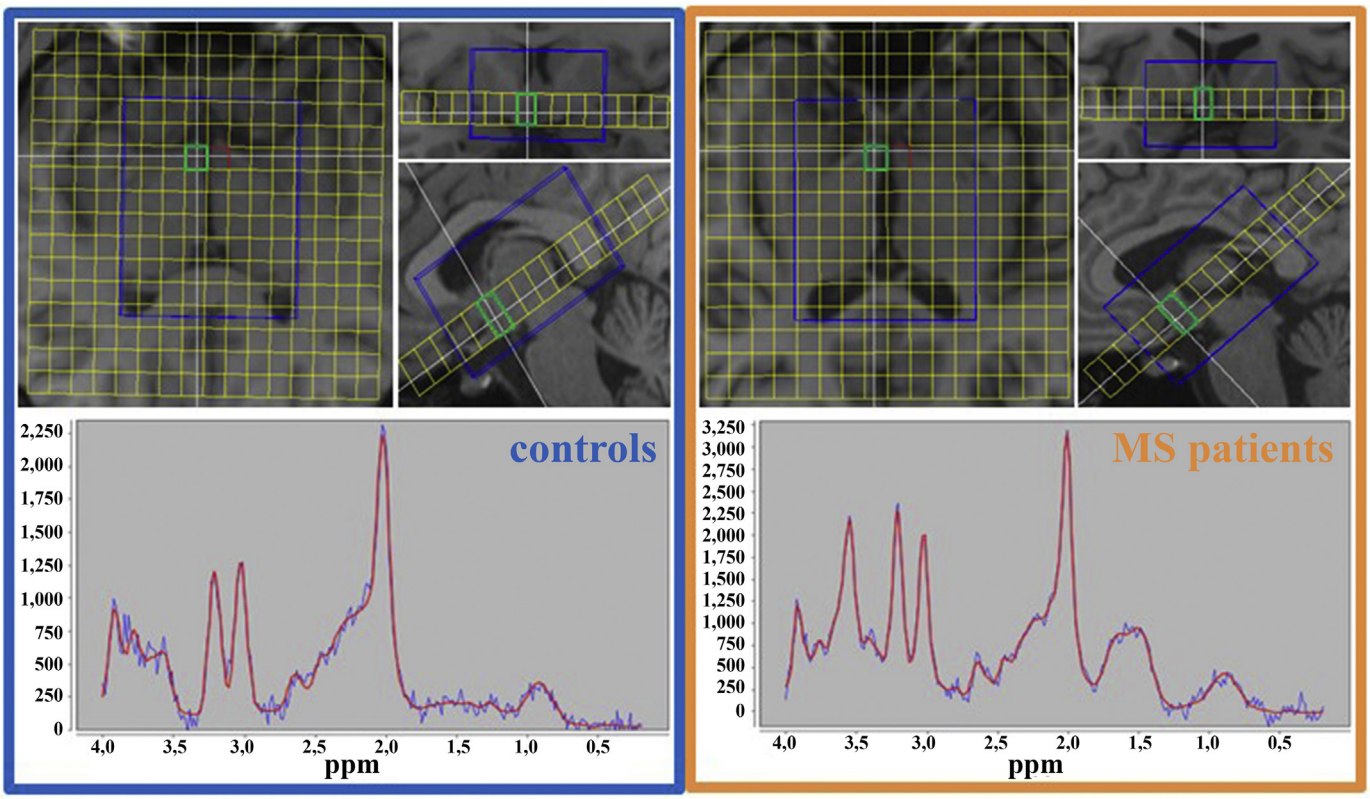

Fig. 2. Evaluation of ^{1}H MRS in hypothalamus of MS patients and controls.
Selection of spectroscopic CSI voxel in the hypothalamic region (green rectangle) based on the overlapping of MP-RAGEs with the CSI matrix in jSIPRO. The spectra of required voxels for healthy controls (in blue) as well as for patients with early MS stages (MS patients; in orange) were evaluated by LCModel.

association in tNAA/tCr and metabolite ratios to tNAA, but not to tCr (Table 4). Test for difference in correlation coefficients when referencing metabolites to tCr or tNAA showed a significant difference only in tCho, not in Glx and mIns (Table 4).

4. Discussion

Our results provide evidence of significant metabolic alterations in the hypothalamus of MS patients with the early stages of the disease, independent of early MS subgroups (CIS vs. CDMS), WM lesion load (≤20 vs. > 20 lesions), disease duration (≤6 vs. > 6 months) or clinical onset (mono- vs. multifocal).

4.1. Decreased tNAA alerts to neuronal damage and increased disability

In our study, tNAA was considered to be one of crucial metabolites in determining disease severity. Since it is known that a reduction in neuronal density directly leads to a decrease in tNAA concentration [17,18,22], we interpret the observed lower tNAA/tCr ratio in the hypothalamus of early MS patients as a loss of neuronal functions and viability and/or neuronal density, even in the early stages of the disease. The results are in accordance with our previous research, where we had found neuro-axonal changes of hypothalamus in correlation with tNAA/tCr of RRMS and SPMS patients [11,12]. Combining our studies, we hypothesize that the existence of hypothalamic neuro-axonal damage (decreased tNAA) is evident from the earliest stages of MS and later becomes the hallmark of DGM damage. The majority of previous reports evaluating other brain structures also confirmed a reduction in tNAA: in acute MS lesions [17,22], in chronic MS lesions [21,23], in NAWM [17,23], in cortical GM, and some structures of DGM in progressive MS stages [21,23,34,35]. There was also confirmed tNAA decline in NAWM and cortical GM in early RRMS [19], in the basal ganglia and thalamus in RRMS [36,37], as well as in the thalamus and hippocampus in primary progressive MS (PPMS) [17,38]. To our knowledge, this is the first study showing hypothalamic damage in patients with early stages of MS, using ^{1}H MRS, with additional EDSS correlation. We observed that the degree of tNAA/tCr reduction in hypothalamus of patients with early stages of MS inversely correlated with increasing neurological disability. Several authors had reported that the highest tNAA loss is most pronounced in those MS patients with greatest neurological disability [21,23,28,39]. However, these studies were almost exclusively focused on the developed MS stage in CNS structures other than the hypothalamus. In this study we confirmed the intensive degeneration of the hypothalamus correlating with disability even in the early stages of MS.

4.2. Elevated Glx reveals neuro-excitotoxicity

Since glutamate plays an important role in nitrogen and energy metabolism and is essential for the complex communication network established between neurons, astrocytes, oligodendrocytes, endothelial and immune cells [3,9,13,14], it is assumed that excessive activation of the glutamatergic pathway could be an important part of MS pathophysiology [9,13,14,16]. In this study, we found increased Glx/tCr as well as Glx/tNAA in the hypothalamus of patients with early stages of MS. Our group have already published elevated Glx ratios in the hypothalamus of RRMS and SPMS patients, with significantly higher levels in active rather than non-active MS [11]. Similarly, an increased Glx level was reported in other CNS structures and MS stages: in NAWM and in active WM lesions of RRMS [14,21,40], SPMS and PPMS patients [16], as well as in NAWM and the thalamus in CDMS patients [22]. However, Glx concentrations were not raised in the chronic WM lesions of RRMS patients [14,40]. Glutamate has, therefore, been proposed as a predictive marker for WM pathology, pointing to neuronal and axonal

Table 2
Hypothalamic ^{1}H MRS in regard to MS subgroups, lesion extension, disease duration, clinical onset, and brain area degeneration.

Groups		Met. ratio						
		tNAA/tCr (mean ± SD)	**tCho/tCr** (mean ± SD)	**tCho/tNAA** (mean ± SD)	**mIns/tCr** (mean ± SD)	**mIns/tNAA** (mean ± SD)	**Glx/tCr** (mean ± SD)	**Glx/tNAA** (mean ± SD)
MS subgroups	**CIS** /*n* = 26/	1.194 ± 0.257	0.348 ± 0.060	0.320 ± 0.189	0.951 ± 0.292	0.784 ± 0.168	2.827 ± 1.045	2.350 ± 0.876
	CDMS /*n* = 36/	1.174 ± 0.264	0.354 ± 0.086	0.307 ± 0.069	0.953 ± 0.310	0.836 ± 0.288	2.288 ± 0.727	1.992 ± 0.612
	***p*-value**	0.764	0.782	0.732#	0.889	0.414	0.102	0.063
Account of WM lesions	**≤20 lesions** /*n* = 34/	1.179 ± 0.267	0.335 ± 0.087	0.288 ± 0.069	0.897 ± 0.289	0.777 ± 0.243	2.416 ± 0.913	2.103 ± 0.805
	> 20 lesions /*n* = 28/	1.186 ± 0.253	0.371 ± 0.051	0.341 ± 0.179	1.018 ± 0.305	0.858 ± 0.243	2.633 ± 0.903	2.190 ± 0.686
	***p*-value**	0.915	0.059	0.065#	0.246	0.197	0.354	0.648
Disease duration	**≤6 months** /*n* = 44/	1.167 ± 0.251	0.352 ± 0.077	0.321 ± 0.153	0.953 ± 0.319	0.814 ± 0.236	2.591 ± 0.979	2.237 ± 0.840
	> 6 months /*n* = 18/	1.219 ± 0.281	0.349 ± 0.073	0.291 ± 0.050	0.949 ± 0.255	0.813 ± 0.270	2.326 ± 0.689	1.909 ± 0.384
	***p*-value**	0.481	0.900	0.215#	0.777	0.994	0.299	0.119
Clinical onset	**mono** /n = 18/	1.086 ± 0.158	0.322 ± 0.084	0.295 ± 0.057	0.878 ± 0.122	0.821 ± 0.143	2.326 ± 0.970	2.157 ± 0.867
	multi /n = 44/	1.222 ± 0.283	0.363 ± 0.070	0.319 ± 0.153	0.982 ± 0.345	0.811 ± 0.278	2.590 ± 0.884	2.136 ± 0.709
	***p*-value**	0.061	0.054	0.085#	0.222	0.878	0.305	0.920
Brain degeneration	**optic neuritis** /n = 26/	1.187 ± 0.252	0.354 ± 0.081	0.328 ± 0.196	0.854 ± 0.347	0.702 ± 0.227	2.433 ± 1.002	2.040 ± 0.857
	brain /n = 36/	1.179 ± 0.267	0.349 ± 0.073	0.301 ± 0.053	1.023 ± 0.242	0.894 ± 0.228	2.571 ± 0.847	2.215 ± 0.668
	***p*-value**	0.900	0.785	0.929#	**0.027**	**0.002**	0.560	0.371

The table shows the values (mean ± standard deviation) of hypothalamic metabolite ratios in MS subgroups (CIS vs. CDMS), WM lesion load (≤20 vs. > 20 lesions), disease duration (≤6 vs. > 6 months), clinical onset (mono- vs. multifocal), and brain region degeneration (optic neuritis vs. brainstem, hemisphere, and spinal cord symptoms). *P*-values express statistical differences in metabolite ratios between both groups with a significance level of $p \leq .05$. For statistical analysis a 2-tailed 2-sample *t*-test for normally distributed data was used, and a Wilcoxon (#) signed-rank test for data that cannot be assumed to be normally distributed.

Table 3
Hypothalamic ^{1}H MRS in early MS patients and controls.

Metabolite ratio	MS patients /n = 62/(mean ± SD)	Controls/n = 62/(mean ± SD)	p-value
tNAA/tCr	1.182 ± 0.259	1.344 ± 0.312	**0.0022**
tCho/tCr	0.351 ± 0.075	0.358 ± 0.082	0.4721
tCho/tNAA	0.312 ± 0.132	0.269 ± 0.056	**0.0056**$^{\#}$
mIns/tCr	0.952 ± 0.300	0.913 ± 0.358	0.3032
mIns/tNAA	0.814 ± 0.245	0.697 ± 0.282	**0.0150**
Glx/tCr	2.513 ± 0.910	2.185 ± 0.960	**0.0235**
Glx/tNAA	2.142 ± 0.751	1.642 ± 0.676	**0.0001**

The table shows the values (mean ± standard deviation) of hypothalamic metabolite ratios in patients with early MS stages (MS patients) and healthy controls. *P*-values express statistical differences in metabolite ratios between both groups with a significance level of $p \leq .05$. For statistical analysis, a 2-tailed 2-sample *t*-test for normally distributed data was used, and a Wilcoxon ($^{\#}$) signed-rank test for data that cannot be assumed to be normally distributed.

damage [14,21]. Findings of Glx in GM are ambiguous: excessive in cortical GM in RRMS [14], unchanged in the thalamus, hippocampus, and cortical GM in RRMS, SPMS, and PPMS [38], or decreased in cortical GM [1], hippocampus, thalamus, cingulate, and parietal cortices in RRMS [40]. It seems that not only the type and location of MS lesions, but also a diffuse neurodegenerative process may influence glutamatergic pathways. It has been demonstrated that elevated glutamate leads to the destruction of oligodendrocytes, which suggests a direct linkage between elevated Glx concentrations and disease progression [15,22,41]. Since glutamate is stored in neurons, which are known to have a reduced function and/or integrity in MS, Glx/tNAA may be a biologically more relevant predictor than either metabolite alone. Our previous research showed a correlation of Glx/tCr and Glx/tNAA ratios with disease severity, measured by Multiple Sclerosis Severity Score in

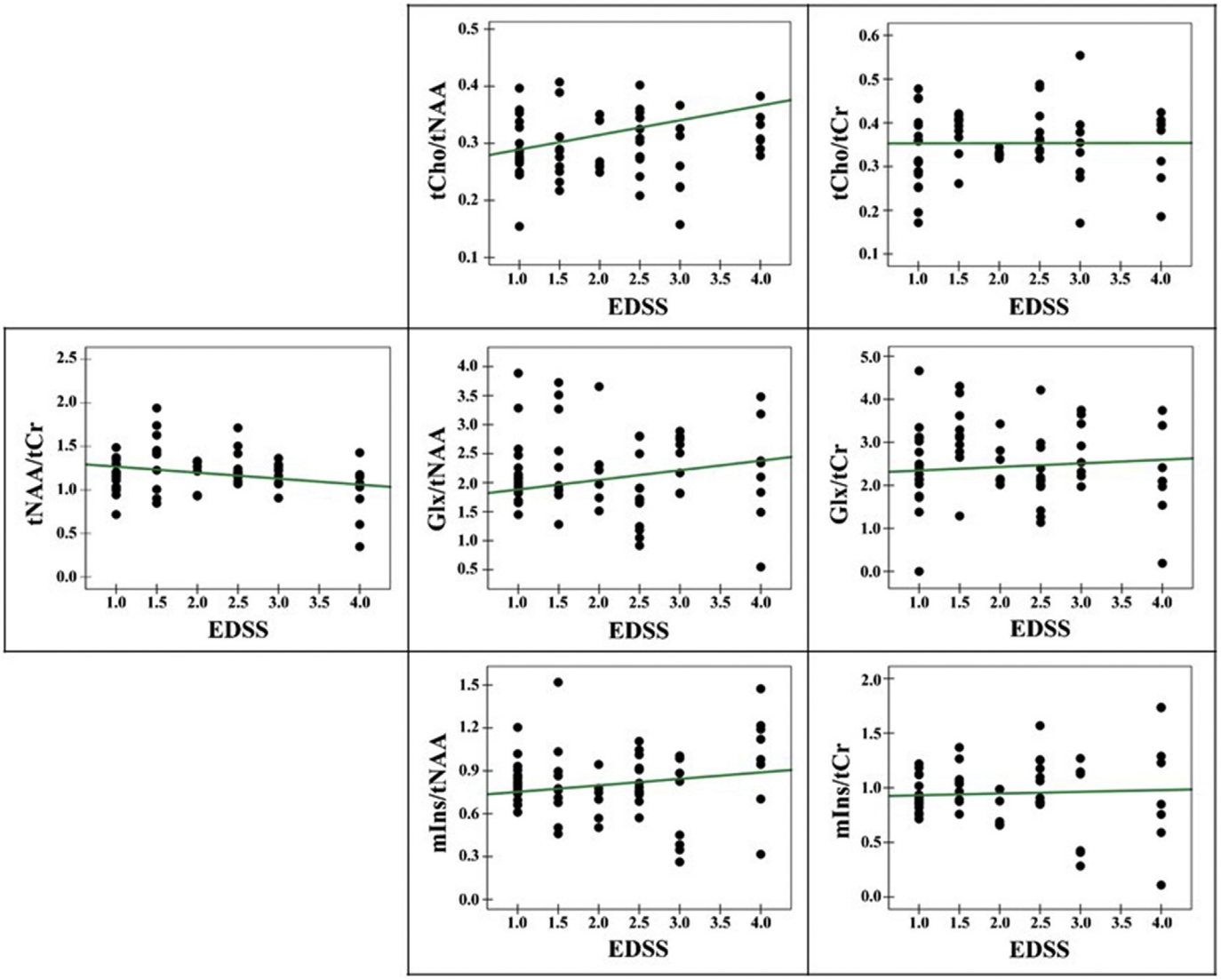

Fig. 3. Pearson correlation plots of metabolite ratios and EDSS.
A graphical depiction of the *Pearson correlation* analysis of hypothalamic metabolite ratios and Expanded Disability Status Scale (EDSS) in patients with early MS stages.

Table 4
Pearson correlation analysis of metabolite ratios and EDSS.

Pearson correlation analysis of metabolite ratios and EDSS			
Metabolite ratio	**r (95% CI lower; upper)**	***p*-value**	***Fisher's p-value***
tNAA/tCr	−0.296 (−0.449; −0.126)	**0.001**	–
tCho/tCr	0.004 (−0.172; 0.180)	0.963	**0.012**
tCho/tNAA	0.317 (0.149; 0.467)	< **0.001**	
Glx/tCr	0.112 (−0.065; 0.283)	0.213	0.173
Glx/tNAA	0.280 (0.109; 0.435)	**0.002**	
mIns/tCr	0.061 (−0.116; 0.235)	0.500	0.221
mIns/tNAA	0.215 (0.040; 0.337)	**0.016**	

The table shows the *Pearson correlation* analysis (r = *Pearson correlation* coefficient; 95% lower and upper CI = confidence interval) and the statistical significance of the linear association (2-tailed *p*-values) of hypothalamic metabolite ratios and Expanded Disability Status Scale (EDSS) in patients with early MS stages. Fisher's r-to-z transformation (Fisher's *p*-value) was used to clarify statistical differences in the correlations of metabolite and EDSS referencing to tCr or tNAA.

RRMS and SPMS patients [11,12]. This study found a significantly positive correlation of Glx/tNAA ratio (but not in Glx/tCr, without statistically significant difference between coefficients) with EDSS in hypothalamus, even in the early disease stages. It indicates that Glx in hypothalamus may be associated with the manifestation of MS, even when no MR-visible lesions are detected in the hypothalamus. Further research is needed to establish whether excessive Glx precedes even the first radiological signs of MS.

4.3. Ratios of mIns and tCho reflect primary neuronal dysfunction

In this study, we evaluated mIns and tCho that are associated with neuroglial homeostasis, cell membrane pathological processes, and ongoing gliosis, which are important signs of MS progression [20,21,25]. However, the hypothalamus of patients with early stages of MS contained significantly increased mIns/tNAA as well as tCho/tNAA, but mIns/tCr or tCho/tCr were not significantly elevated.

The metabolites mIns and tCr are suggested to be glial cell markers [21–23] and tNAA to be a neuronal marker [20,22,28]. In this light, our results indicate a simultaneous increase in glial cells with a decrease in neurons in the hypothalamus of early MS patients. As far as we know, we are the only group focusing on the hypothalamic region in depth, confirming higher mIns/tNAA ratio not only in early stages of MS, but also in RRMS and SPMS [11,12]. Our entire research suggests that gliosis (increased mIns) appears from the early to the progressive stages of MS. This is in accordance with other authors evaluating different GM regions, who have found elevated mIns in cortical GM in RRMS [21], and in the thalamus and hippocampus in RRMS, SPMS, and PPMS [38]. Increased mIns was detected also in other CNS regions at various stages of the disease: in acute WM lesions and NAWM of RRMS, SPMS, and PPMS patients [16,21,25], as well as in NAWM of CIS patients [26,27]. In addition, some authors consider mIns/tNAA evaluating in WM as a predictor of clinical disability in MS [18,27]. Regarding the brain regions' degeneration (optic neuritis vs. brainstem, hemisphere, and spinal cord symptoms) in MS patients included in this study, the only alteration in hypothalamic metabolism was confirmed in mIns and mIns/tCr. Both mIns ratios were significantly lower in MS patients with optic neuritis compared to the MS patients with neurodegeneration in other brain areas. Optic neuritis is an inflammatory condition of the afferent visual system and although is strongly related to MS, it may be associated with a variety of autoimmune or infectious etiologies [42–44]. According to our results, it seems that ongoing inflammation and gliosis during this specific condition are more WM tract (i.e. retinohypothalamic tract) restricted and/or have lower extent of DGM neuro-axonal degeneration [10], compared to brainstem, hemisphere, or spinal cord symptoms. This could have an impact on reducing the risk of MS developing into optic neuritis [42,44]. Several studies suggested that in the early phases of the disease, the neuro-axonal damage in WM may be at least partially reversible [21,27,41]. This support the theory of the dual role of astrocytes in MS. They may contribute to the degeneration of oligodendrocytes and axons' demyelination by promoting inflammation and on the other side, they may create a permissive environment for remyelination [9,18,43].

Considering tCho as a marker of cell membrane and myelin sheet integrity [13,20,21], the increased tCho/tNAA found in our study showed intensive cell-membrane and myelin degradation and/or turnover, parallel to hypothalamic neurodegeneration in our early MS patients. We hypothesize that in early MS stages, brain DGM is under strong inflammatory/neuroexcitatory influence, causing a loss of hypothalamic cells which decreases tNAA. Cell-membrane and myelin-sheet breakdown products accumulate in the area and thus increase tCho. The brain metabolism is overloaded and the brain is forced to regenerate impaired GM tissue, leading to a simultaneous increase in tCho [21,26]. In contrast with this study, our previous research focusing on RRMS and SPMS patients, with a longer disease duration, revealed lower tCho/tCr ratios in the hypothalamus compared with controls [11,12]. Combining our studies, we hypothesize that the initial hypothalamic active demyelination (increased tCho) in progressive MS stages is evidently absents (decreased tCho). This is in agreement with a study of patients with CIS, showing an increased Cho level in the thalamus [21,29]. In other reports, no differences in tCho concentrations were found in the thalamus, hippocampus, and cortical GM of RRMS, but tCho decreased in progressive MS stages [35,38]. However, the hypothalamic region was not tested. Other authors reported variable tCho levels in cortical and subcortical GM and interpreted it as fluctuations in membrane turnover rates due to the inflammation/demyelination processes [21,26,29,36,38].

In our study, it was also found that tCho/tNAA as well as mIns/tNAA (but not ratios to tCr) in the hypothalamus of patients with early stages of MS significantly positively correlated with EDSS. In addition, a test for differences in correlation with EDSS when referring to metabolites to tCr or tNAA revealed a significant difference in tCho ratios, showing the predictive importance of tCho/tNAA in early MS evolution. This indicates that progressive neural loss together with gliosis in the hypothalamus reflect the disease's severity, even in the early stages of MS. It also seems that alterations of mIns and tCho in the hypothalamus of early MS patients result from primary neuronal dysfunction. However, an increase in tCho in the hypothalamic region could also be caused by a Wallerian degeneration of tracts connecting DGM with WM [45,46]. On the other hand, the accumulation of glial cells, expressed as an increase in mIns, suggests the relatively intensive replacement of neurons via glial tissue even in the early stages of MS, which supports the superiority of primary neurodegeneration over inflammatory processes [8,11,34,43]. This is in agreement with recent findings of non-immune-related neurotoxicity in GM, based on the dysfunction of glutamate circuits leading to excitatory damage [14].

4.4. Study limitations

The findings of our study should be interpreted in the context of several limitations, such as the relatively small sample size, the lack of information about hypothalamic functions (e.g. hypothalamic hormone levels, fatigue, sleep disorders, mental disorders) across both patients and control participants, and the short design of the study without longitudinal MS monitoring. Furthermore, the hypothalamus is a challenging brain area for ^{1}H MRS acquisition due to its location close to the confounding air- and CSF-rich regions, as well as due to its small volume prone to atrophy [31–33]. Therefore, technical factors like 1.5 T MR-scanner application, potential patient movement during the acquisition, quality of shimming, and the reproducibility of voxel location on subsequent measurements may also represent potential confounders. On the other hand, almost large GM nuclei in the

hypothalamic perimeter do not contaminate the ^{1}H MRS signal as much as it can be assumed for the other DGM brain regions. Finally, the relative metabolite quantification used in the form of metabolite ratios may be less straightforward compared to absolute ^{1}H MRS approach, but previous studies have shown the good informative value of this method [17,18,23].

5. Conclusion

Our study is one of only a few advanced MR studies examining the nature of the hypothalamus in MS. Results provide evidence of significant hypothalamic metabolic alterations that correlate with EDSS in the very early stages of the disease. Based on the alterations of metabolic ratios, we hypothesize that the early glutamate-induced neurodegeneration of hypothalamus can lead to neuronal loss paralleled by the decline of tNAA. The combination of increased mIns and tCho, together with reduced tNAA in the hypothalamus, possibly reflects the microglial activation and degradation of myelinated tracts. We also found a different metabolic background in the hypothalamus of patients with optic neuritis when compared with other early MS patients. The presented results further extend the data provided by previous research, however the pathophysiology of the hypothalamic metabolic compartments needs to be more precisely quantified by in vitro methods and validated by longitudinal studies.

Acknowledgement

This work was supported by the Biomedical Center Martin project (ITMS 2622 0220187) and by the grants from the Slovak Research and Development Agency (VEGA 1/0301/19) and the Scientific Grant Agency of the Ministry of Education, Science, Research and Sport of the Slovak Republic and the Slovak Academy of Sciences (APVV-15-0107, APVV-14-0088), co-funded from EU sources.

References

[1] S. Messina, F. Patti, Gray matters in multiple sclerosis: cognitive impairment and structural MRI, Mult. Scler. Int. 2014 (2014) 609694, , https://doi.org/10.1155/2014/609694.
[2] M. Donadieu, Y. Le Fur, A. Lecocq, A.A. Maudsley, S. Gherib, E. Soulier, S. Confort-Gouny, F. Pariollaud, M.P. Ranjeva, J. Pelletier, M. Guye, W. Zaaraoui, B. Audoin, J.P. Ranjeva, Metabolic voxel-based analysis of the complete human brain using fast 3D-MRSI: proof of concept in multiple sclerosis, J. Magn. Reson. Imaging 44 (2) (2016) 411–419, https://doi.org/10.1002/jmri.25139.
[3] R. Dutta, J. McDonough, X. Yin, J. Peterson, A. Chang, T. Torres, T. Gudz, W.B. Macklin, D.A. Lewis, R.J. Fox, R. Rudick, K. Mirnics, B.D. Trapp, Mitochondrial dysfunction as a cause of axonal degeneration in multiple sclerosis patients, Ann. Neurol. 59 (3) (2006) 478–489, https://doi.org/10.1002/ana.20736.
[4] I.S. Haussleiter, M. Brune, G. Juckel, Psychopathology in multiple sclerosis: diagnosis, prevalence and treatment, Ther. Adv. Neurol. Disord. 2 (1) (2009) 13–29, https://doi.org/10.1177/1756285608100325.
[5] K.G. Burfeind, V. Yadav, D.L. Marks, Hypothalamic dysfunction and multiple sclerosis: implications for fatigue and weight dysregulation, Curr. Neurol. Neurosci. Reports 16 (11) (2016) 98, https://doi.org/10.1007/s11910-016-0700-3.
[6] N. Bergsland, D. Horakova, M.G. Dwyer, O. Dolezal, Z.K. Seidl, M. Vaneckova, J. Krasensky, E. Havrdova, R. Zivadinov, Subcortical and cortical gray matter atrophy in a large sample of patients with clinically isolated syndrome and early relapsing-remitting multiple sclerosis, AJNR Am. J. Neuroradiol. 33 (8) (2012) 1573–1578, https://doi.org/10.3174/ajnr.A3086.
[7] R. Zivadinov, M. Heininen-Brown, C.V. Schirda, G.U. Poloni, N. Bergsland, C.R. Magnano, J. Durfee, C. Kennedy, E. Carl, J. Hagemeier, R.H. Benedict, B. Weinstock-Guttman, M.G. Dwyer, Abnormal subcortical deep-gray matter susceptibility-weighted imaging filtered phase measurements in patients with multiple sclerosis: a case-control study, NeuroImage 59 (1) (2012) 331–339, https://doi.org/10.1016/j.neuroimage.2011.07.045.
[8] D. Horakova, T. Kalincik, J.B. Dusankova, O. Dolezal, Clinical correlates of grey matter pathology in multiple sclerosis, BMC Neurol. 12 (2012) 10, https://doi.org/10.1186/1471-2377-12-10.
[9] N. Deckx, W.P. Lee, Z.N. Berneman, N. Cools, Neuroendocrine immunoregulation in multiple sclerosis, Clin. Dev. Immunol. 2013 (2013) 705232, , https://doi.org/10.1155/2013/705232.
[10] I. Huitinga, C.J. De Groot, P. Van der Valk, W. Kamphorst, F.J. Tilders, D.F. Swaab, Hypothalamic lesions in multiple sclerosis, J. Neuropathol. Exp. Neurol. 60 (12) (2001) 1208–1218.
[11] E. Kantorova, H. Polacek, M. Bittsansky, E. Baranovicova, P. Hnilicova, D. Cierny, S. Sivak, V. Nosal, K. Zelenak, E. Kurca, Hypothalamic damage in multiple sclerosis correlates with disease activity, disability, depression, and fatigue, Neurol. Res. 39 (4) (2017) 323–330, https://doi.org/10.1080/01616412.2016.1275460.
[12] H. Polacek, E. Kantorova, P. Hnilicova, M. Grendar, K. Zelenak, E. Kurca, Increased glutamate and deep brain atrophy can predict the severity of multiple sclerosis, Biomed. Pap. Med. Fac. Univ. Palacky Olomouc Czech Repub. 163 (1) (2019) 45–53, https://doi.org/10.5507/bp.2018.036.
[13] I.R. Stojanovic, M. Kostic, S. Ljubisavljevic, The role of glutamate and its receptors in multiple sclerosis, J. Neural Transm. 121 (8) (2014) 945–955, https://doi.org/10.1007/s00702-014-1188-0.
[14] R. Macrez, P.K. Stys, D. Vivien, S.A. Lipton, F. Docagne, Mechanisms of glutamate toxicity in multiple sclerosis: biomarker and therapeutic opportunities, Lancet Neurol. 15 (10) (2016) 1089–1102, https://doi.org/10.1016/S1474-4422(16)30165-X.
[15] C.J. Azevedo, J. Kornak, P. Chu, M. Sampat, D.T. Okuda, B.A. Cree, S.J. Nelson, S.L. Hauser, D. Pelletier, In vivo evidence of glutamate toxicity in multiple sclerosis, Ann. Neurol. 76 (2) (2014) 269–278, https://doi.org/10.1002/ana.24202.
[16] R. Srinivasan, N. Sailasuta, R. Hurd, S. Nelson, D. Pelletier, Evidence of elevated glutamate in multiple sclerosis using magnetic resonance spectroscopy at 3 T, Brain 128 (2005) 1016–1025, https://doi.org/10.1093/brain/awh467 Pt 5.
[17] N. De Stefano, M.L. Bartolozzi, L. Guidi, M.L. Stromillo, A. Federico, Magnetic resonance spectroscopy as a measure of brain damage in multiple sclerosis, J. Neurol. Sci. 233 (1–2) (2005) 203–208, https://doi.org/10.1016/j.jns.2005.03.018.
[18] S. Llufriu, J. Kornak, H. Ratiney, J. Oh, D. Brenneman, B.A. Cree, M. Sampat, S.L. Hauser, S.J. Nelson, D. Pelletier, Magnetic resonance spectroscopy markers of disease progression in multiple sclerosis, JAMA Neurol. 71 (7) (2014) 840–847, https://doi.org/10.1001/jamaneurol.2014.895.
[19] M. Tiberio, D.T. Chard, D.R. Altmann, G. Davies, C.M. Griffin, M.A. McLean, W. Rashid, J. Sastre-Garriga, A.J. Thompson, D.H. Miller, Metabolite changes in early relapsing-remitting multiple sclerosis - a two year follow-up study, J. Neurol. 253 (2) (2006) 224–230, https://doi.org/10.1007/s00415-005-0964-z.
[20] D.P. Soares, M. Law, Magnetic resonance spectroscopy of the brain: review of metabolites and clinical applications, Clin. Radiol. 64 (1) (2009) 12–21, https://doi.org/10.1016/j.crad.2008.07.002.
[21] B.R. Sajja, J.S. Wolinsky, P.A. Narayana, Proton magnetic resonance spectroscopy in multiple sclerosis, Neuroimaging Clin. N. Am. 19 (1) (2009) 45–58, https://doi.org/10.1016/j.nic.2008.08.002.
[22] A. Tisell, O.D. Leinhard, J.B. Warntjes, A. Aalto, O. Smedby, A.M. Landtblom, P. Lundberg, Increased concentrations of glutamate and glutamine in normal-appearing white matter of patients with multiple sclerosis and normal MR imaging brain scans, PLoS ONE 8 (4) (2013) e61817, , https://doi.org/10.1371/journal.pone.0061817.
[23] J.L. Ruiz-Pena, P. Pinero, G. Sellers, J. Argente, A. Casado, J. Foronda, A. Ucles, G. Izquierdo, Magnetic resonance spectroscopy of normal appearing white matter in early relapsing-remitting multiple sclerosis: correlations between disability and spectroscopy, BMC Neurol. 4 (8) (2004), https://doi.org/10.1186/1471-2377-4-8.
[24] W.H. Zaini, F. Giuliani, C. Beaulieu, S. Kalra, C. Hanstock, Fatigue in multiple sclerosis: assessing pontine involvement using proton MR spectroscopic imaging, PLoS ONE 11 (2) (2016) e0149622, , https://doi.org/10.1371/journal.pone.0149622.
[25] M. Quarantelli, MRI/MRS in neuroinflammation: methodology and applications, Clin. Transl. Imaging 3 (2015) 475–489, https://doi.org/10.1007/s40336-015-0142-y.
[26] I.I. Kirov, A. Tal, J.S. Babb, J. Herbert, O. Gonen, Serial proton MR spectroscopy of gray and white matter in relapsing-remitting MS, Neurology 80 (1) (2013) 39–46, https://doi.org/10.1212/WNL.0b013e31827b1a8c.
[27] K.T. Fernando, M.A. McLean, D.T. Chard, D.G. MacManus, C.M. Dalton, K.A. Miszkiel, R.M. Gordon, G.T. Plant, A.J. Thompson, D.H. Miller, Elevated white matter myo-inositol in clinically isolated syndromes suggestive of multiple sclerosis, Brain 127 (2004) 1361–1369, https://doi.org/10.1093/brain/awh153 Pt 6.
[28] J. Gillard, A. Wladman, P. Barker, Clinical MR Neuroimaging, Cambridge University Press, Cambridge, 2010.
[29] M.V.A. Duong, B. Audoin, Y. Le Fur, S. Confort-Gouny, I. Malikova, E. Soulier, P. Viout, A. Ali-Cherif, J. Pelletier, P.J. Cozzone, J.P. Ranjeva, Relationships between gray matter metabolic abnormalities and white matter inflammation in patients at the very early stage of MS - a MRSI study, J. Neurol. 254 (7) (2007) 914–923, https://doi.org/10.1007/s00415-006-0474-7.
[30] C.H. Polman, S.C. Reingold, B. Banwell, M. Clanet, J.A. Cohen, M. Filippi, K. Fujihara, E. Havrdova, M. Hutchinson, L. Kappos, F.D. Lublin, X. Montalban, P. O'Connor, M. Sandberg-Wollheim, A.J. Thompson, E. Waubant, B. Weinshenker, J.S. Wolinsky, Diagnostic criteria for multiple sclerosis: 2010 revisions to the McDonald criteria, Ann. Neurol. 69 (2) (2011) 292–302, https://doi.org/10.1002/ana.22366.
[31] A. Moheet, U.E. Emir, M. Terpstra, A. Kumar, L.E. Eberly, E.R. Seaquist, G. Oz, Initial experience with seven tesla magnetic resonance spectroscopy of hypothalamic GABA during hyperinsulinemic euglycemia and hypoglycemia in healthy humans, Magn. Reson. Med. 71 (1) (2014) 12–18, https://doi.org/10.1002/mrm.24663.
[32] J.M. Joers, D.K. Deelchand, A. Kumar, A. Moheet, E. Seaquist, P.G. Henry, G. Oz, Measurement of hypothalamic glucose under Euglycemia and hyperglycemia by MRI at 3T, J. Magn. Reson. Imaging 45 (3) (2017) 681–691, https://doi.org/10.1002/jmri.25383.
[33] S.J. van den Bogaard, E.M. Dumas, W.M. Teeuwisse, H.E. Kan, A. Webb, R.A. Roos, J. van der Grond, Exploratory 7-tesla magnetic resonance spectroscopy in Huntington's disease provides in vivo evidence for impaired energy metabolism, J. Neurol. 258 (12) (2011) 2230–2239, https://doi.org/10.1007/s00415-011-6099-5.

[34] H.E. Hulst, J.J. Geurts, Gray matter imaging in multiple sclerosis: what have we learned? BMC Neurol. 11 (2011) 153, https://doi.org/10.1186/1471-2377-11-153.
[35] M. Filippi, M.A. Rocca, MR imaging of gray matter involvement in multiple sclerosis: implications for understanding disease pathophysiology and monitoring treatment efficacy, AJNR Am. J. Neuroradiol. 31 (7) (2010) 1171–1177, https://doi.org/10.3174/ajnr.A1944.
[36] M. Inglese, S. Liu, J.S. Babb, L.J. Mannon, R.I. Grossman, O. Gonen, Three-dimensional proton spectroscopy of deep gray matter nuclei in relapsing-remitting MS, Neurology 63 (1) (2004) 170–172.
[37] M. Wylezinska, A. Cifelli, P. Jezzard, J. Palace, M. Alecci, P.M. Matthews, Thalamic neurodegeneration in relapsing-remitting multiple sclerosis, Neurology 60 (12) (2003) 1949–1954.
[38] J.J. Geurts, I.E. Reuling, H. Vrenken, B.M. Uitdehaag, C.H. Polman, J.A. Castelijns, F. Barkhof, P.J. Pouwels, MR spectroscopic evidence for thalamic and hippocampal, but not cortical, damage in multiple sclerosis, Magn. Reson. Med. 55 (3) (2006) 478–483, https://doi.org/10.1002/mrm.20792.
[39] O. Khan, N. Seraji-Bozorgzad, F. Bao, S. Razmjou, C. Caon, C. Santiago, Z. Latif, R. Aronov, I. Zak, N. Ashtamker, S. Kolodny, C. Ford, Y. Sidi, The relationship between brain MR spectroscopy and disability in multiple sclerosis, J. Neuroimaging 27 (1) (2017) 97–106.
[40] N. Muhlert, M. Atzori, E. De Vita, D.L. Thomas, R.S. Samson, C.A.M. Wheeler-Kingshott, J.J.G. Geurts, D.H. Miller, A.J. Thompson, O. Ciccarelli, Memory in multiple sclerosis is linked to glutamate concentration in grey matter regions, J. Neurol. Neurosurg. Psychiatry 85 (8) (2014) 834–840, https://doi.org/10.1136/jnnp-2013-306662.
[41] N. Jafari, Risk Factors in Cause and Course of Multiple Sclerosis, Ridderprint BV, Amsterdam, 2011.
[42] E.V. Burton, B.M. Greenberg, E.M. Frohman, Optic neuritis: a mechanistic view, Pathophysiology 18 (1) (2011) 81–92, https://doi.org/10.1016/j.pathophys.2010.04.009.
[43] S.L. Hauser, J.R. Oksenberg, The neurobiology of multiple sclerosis: genes, inflammation, and neurodegeneration, Neuron 52 (1) (2006) 61–76, https://doi.org/10.1016/j.neuron.2006.09.011.
[44] E. Voss, P. Raab, C. Trebst, M. Stangel, Clinical approach to optic neuritis: pitfalls, red flags and differential diagnosis, Ther. Adv. Neurol. Disord. 4 (2) (2011) 123–134, https://doi.org/10.1177/1756285611398702.
[45] L. Haider, C. Simeonidou, G. Steinberger, S. Hametner, N. Grigoriadis, G. Deretzi, G.G. Kovacs, A. Kutzelnigg, H. Lassmann, J.M. Frischer, Multiple sclerosis deep grey matter: the relation between demyelination, neurodegeneration, inflammation and iron, J. Neurol. Neurosurg. Psychiatry 85 (12) (2014) 1386–1395, https://doi.org/10.1136/jnnp-2014-307712.
[46] B. Casanova, M.C. Martinez-Bisbal, C. Valero, B. Celda, L. Marti-Bonmati, A. Pascual, L. Landente, F. Coret, Evidence of Wallerian degeneration in normal appearing white matter in the early stages of relapsing-remitting multiple sclerosis - A HMRS study, J. Neurol. 250 (1) (2003) 22–28, https://doi.org/10.1007/s00415-003-0928-0.