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Cortical glutamate in migraine

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Cortical hyperexcitability due to enhanced glutamatergic activity has been implicated in migraine pathophysiology but direct evidence is lacking. Here we assessed glutamate levels and intracellular mobility of glutamate in the visual cortex of migraineurs in-between attacks. We included 50 migraineurs (23 with aura and 27 without aura) and 24 age- and gender-matched non-headache controls. We used proton magnetic resonance spectroscopy (^{1}H-MRS) and diffusion weighted spectroscopy at 7 T with a single volume of interest (2 × 2 × 3 cm) located in the primary and secondary visual cortex. For ^{1}H-MRS we used a semi-LASER sequence with water referencing for absolute quantification. For diffusion weighted spectroscopy we used an adapted PRESS sequence with gradients applied in three directions and two different gradient amplitudes. Between-group differences were evaluated using analysis of covariance with the grey matter fraction in the volume of interest as covariate and *post hoc* comparisons with Bonferroni correction. Glutamate concentrations differed between groups ($P = 0.047$) and were higher in migraineurs without aura (mean ± standard deviation: 7.02 ± 0.50 mM) compared to controls (mean ± standard deviation: 6.40 ± 0.78 mM, $P = 0.042$). The apparent diffusion coefficient of glutamate was similar among groups ($P = 0.129$) suggesting similar inter- and intracellular mobility of glutamate in all three study groups. No differences were observed for concentrations and diffusion constants of other metabolites. The present study suggests that interictal glutamate levels are increased in the visual cortex of migraineurs without aura, supporting the hypothesis of cortical hyperexcitability in migraine.

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Abbreviations: ADC = apparent diffusion coefficient; CRLB = Cramér-Rao lower bound; CSD = cortical spreading depolarization; DWS = diffusion weighted spectroscopy; ^{1}H-MRS = proton magnetic resonance spectroscopy; HIT-6 = headache impact test; NAA = *N*-acetylaspartate

Introduction

Migraine is a common (Jensen and Stovner, 2008) and highly disabling (Global Burden of Disease Study 2013 Collaborators, 2015) episodic brain disorder, typically characterized by recurring attacks of severe headache and accompanying autonomic features for up to 3 days (migraine without aura) [Goadsby *et al.*, 2002; Headache Classification Committee of the International Headache Society (IHS), 2013]. In a third of patients, attacks may

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be associated with a, usually visual, migraine aura (migraine with aura) (Launer *et al.*, 1999), which likely is caused by cortical spreading depolarization (CSD) (for review see Ferrari *et al.*, 2015).

The mechanisms for the triggering, initiation and recurrence of migraine attacks are poorly understood. An important role has been postulated for increased brain glutamatergic activity leading to increased cerebral excitability and enhanced susceptibility to CSD, similar to what has been found for hemiplegic migraine (Bolay 2012; Cosentino *et al.*, 2014; Ferrari *et al.*, 2015). Hemiplegic migraine is a rare subtype of migraine with aura (Headache Classification Committee of the IHS, 2013) and considered a good monogenic model for pathogenetic mechanisms potentially involved in the common, multifactorial, forms of migraine (Ferrari *et al.*, 2015). Mutations in three different genes that have been associated with hemiplegic migraine all lead via different pathways ultimately to net increased cerebral glutamatergic neuroexcitatory activity and enhanced susceptibility to CSD. Whether similar mechanisms are involved in the common forms of migraine is uncertain. Indirect support for this intriguing hypothesis stems from observations that glutamate levels were increased in CSF of patients with chronic migraine and in blood of patients with episodic migraine (van Dongen *et al.*, 2017). In addition, anti-glutamatergic anti-epileptic drugs have shown prophylactic efficacy in migraine with and without aura (Vikelis and Rapoport, 2010). Direct evidence for enhanced glutamatergic activity in the common forms of migraine is, however, still lacking.

Studies attempting to more directly demonstrate increased brain glutamate *in vivo* by using proton magnetic resonance spectroscopy (^{1}H-MRS) have consistently failed, most likely because they were using low magnetic field strengths and protocols that were not optimized to reliably distinguish glutamate from its major precursor glutamine (Dichgans *et al.*, 2005; Prescot *et al.*, 2009; Reyngoudt *et al.*, 2012; Siniatchkin *et al.*, 2012; González *et al.*, 2013; Zielman *et al.*, 2014; Bridge *et al.*, 2015; Arngrim *et al.*, 2016). These studies could thus only report the combined signal of glutamate and glutamine rather than the individual levels. This is a major limitation as both amino acids, although metabolically closely related via the glutamate-glutamine cycle, have very distinct functions and compartmental distributions within the brain. While glutamate is mainly located intracellularly (Danbolt, 2001) in glutamatergic neurons (Erecinska and Silver, 1990), glutamine is primarily found in astrocytes (Erecinska and Silver, 1990).

When assessing glutamate in the brain, it is also important to consider its location within different tissue compartments. The glutamate signal in ^{1}H-MRS and diffusion weighted spectroscopy (DWS) reflects the intracellular pool (about 80% neuronal and 20% astrocytic) of glutamate and not the extracellular or synaptic glutamate (Erecinska and Silver, 1990). Not only the concentration but also the intracellular and subcellular compartmentalization of glutamate (e.g. in synaptic vesicles or in the cytosol) is important for normal brain function (Greenamyre and Porter, 1994). Insufficient compartmentalization might also be an important factor in migraine, because abnormally distributed intracellular glutamate may also have an effect on glutamate metabolism and function. DWS measures the diffusion properties of brain metabolites (Nicolay *et al.*, 2001; Kan *et al.*, 2012), and has been shown to be sensitive to subtle microstructural changes of the intracellular compartments (Wood *et al.*, 2012; Zheng *et al.*, 2012). DWS might thus be helpful in identifying differences in compartmentalization of glutamate between migraineurs and controls.

In the present study, we assessed and compared glutamate levels in the visual cortex of interictal migraineurs with aura, migraineurs without aura, and age- and sex-matched non-headache control subjects. We used ^{1}H-MRS at 7 T and dedicated optimized techniques enabling measuring cerebral glutamate and glutamine separately (Snyder and Wilman, 2010). Moreover, we used DWS to measure the diffusion properties, mobility and, indirectly, intracellular compartmentalization of glutamate (Nicolay *et al.*, 2001; Kan *et al.*, 2012). We preselected the visual cortex as the region of primary interest because previous studies have suggested that this brain structure is hyper-responsive in migraineurs and, in transgenic migraine mouse models, revealed enhanced susceptibility to CSD (Aurora and Wilkinson, 2007; Charles and Baca, 2013).

Materials and methods

Experimental design

We included three age- and sex-matched study populations: migraine without aura ($n = 36$), migraine with aura ($n = 27$) and non-headache healthy controls ($n = 27$). Migraine was diagnosed according to the International Classification of Headache Disorders third edition (beta-version) (Headache Classification Committee of the IHS, 2013). Participants with migraine were included if they had at least one migraine attack per month in the preceding 6 months and did not have chronic migraine or overuse of acute headache medication (Headache Classification Committee of the IHS, 2013). Participants with migraine with aura had to have visual auras in at least half of their attacks. Migraineurs were otherwise healthy without other known medical conditions and refrained from using prophylactic medication for at least 4 weeks and from using acute migraine attack medication for at least 3 days prior to the investigation. Non-headache healthy controls did not have any form of primary or secondary headache, did not have more than one (non-specific) headache day per 3 months, and were otherwise healthy without known neurological or psychiatric disorders. MRS/DWS scans were to be performed interictal, defined as at least 3 days after a previous attack and at least 2 days before a subsequent attack (as checked afterwards). Scans performed within 2 days before a subsequent attack were defined as preictal. All study participants used no chronic medication other than oral contraceptives. The study was approved by the Leiden University Medical Center

institutional ethics committee and conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all subjects prior to the study.

MRS and DWS data acquisition

All magnetic resonance scans were performed before 12.00 p.m. to minimize bias due to diurnal differences. To control for any dietary effect, subjects were asked not to eat or drink anything but water for at least 8 h before the scan. To reduce visual stimulation of the visual cortex, we instructed the subjects to keep their eyes closed throughout the whole scan session.

Subjects were examined at a 7 T magnetic resonance system (Philips) using a 32 channel receive array and a quadrature transmit coil (Nova Medical) driven by two amplifiers (4 kW each) with a fixed phase setting between the two amplifiers to reach a local transmit field (B_1) of 20 μT in the regions of interest. An anatomical 3D T_1-weighted gradient echo image was acquired to allow accurate planning of the volume of interest for MRS and DWS (Fig. 1A and B). Imaging parameters were: field of view: 248 × 246 × 174 mm^3, resolution 1 × 1 × 1 mm^3, repetition time/echo time/inversion time = 4.0/1.81/1300 ms.

For MRS we used a single-volume proton MRS (^{1}H-MRS) semi-localized by adiabatic selective refocusing (semi-LASER)

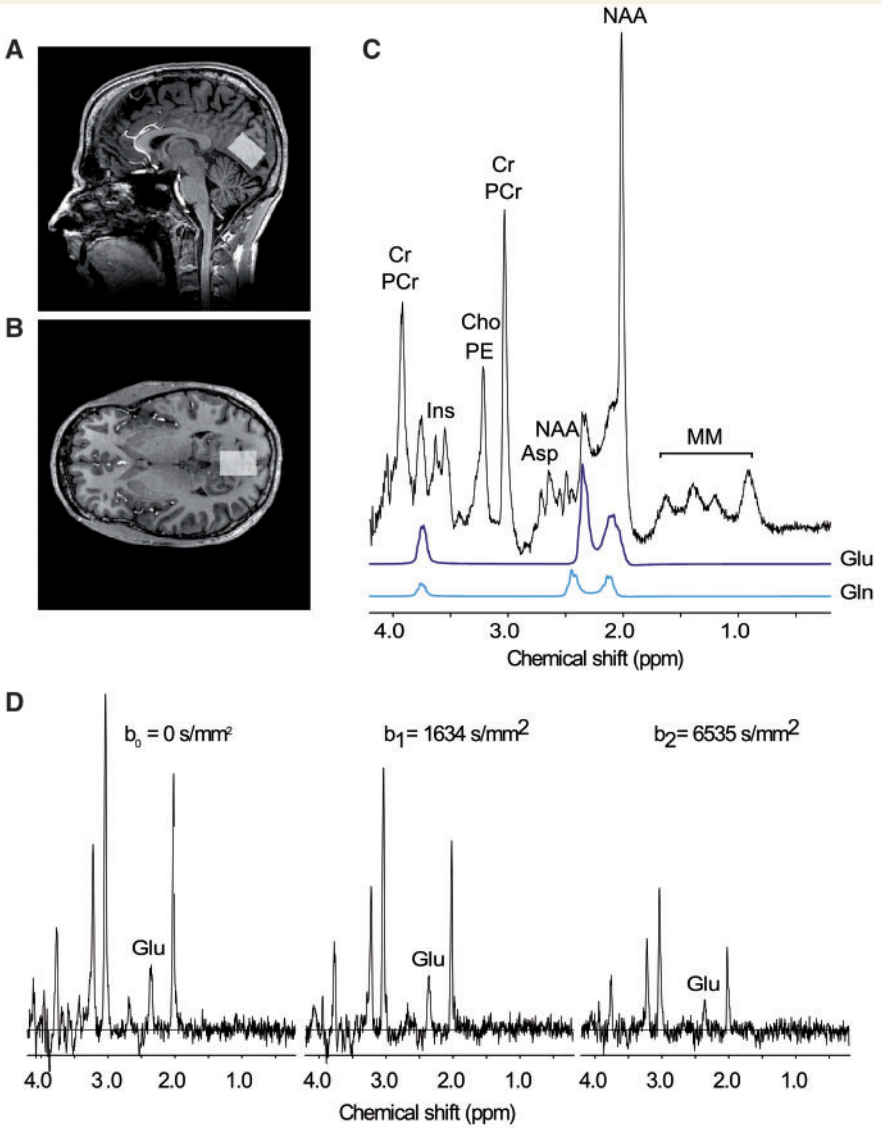

Figure 1 Example data from a male migraine without aura patient. (**A** and **B**) T_1-weighted images in the mid-sagittal and transverse planes, the white box indicates the location of the volume of interest in the occipital lobe with a size of 20 × 20 × 30 mm. (**C**) Example of a ^{1}H-MR spectrum showing the individual fits for glutamate (Glu) and glutamine (Gln), illustrating the fact that glutamate and glutamine do not generate a prominent single peak but rather three distinct peaks; for visual representation the baseline fit was subtracted from the Glu and Gln fit. Asp = aspartate; Cho = choline; Cr = creatine; Ins = myo-inositol; MM = macromolecules; PCr = phosphocreatine; PE = phosphorylethanolamine. (**D**) Averaged ^{1}H-MRS spectra at three different b-values for one diffusion-weighted direction.

sequence (repetition time/echo time = 5000/30 ms, spectral width = 4 kHz, 2048 points, acquisition time ≈4 min) (Boer *et al.*, 2011), preceded by a variable power and optimized relaxation delays (VAPOR) water suppression sequence. The transmitter frequency was set on 2.35 ppm (glutamate). The volume of interest ($30 \times 20 \times 20$ mm^3) was manually planned. The investigator (R.Z.) who included the participants during data acquisition also operated the MRI scanner and was also responsible for placement of the volumes of interest. To avoid bias, clear anatomical landmarks for placement of the volumes of interest were used. The volumes of interest were centred along the calcarine fissure, symmetrically covering both hemispheres caudal of the parieto-occipital fissure and including the primary and secondary visual cortices (Brodmann areas 17 and 18; Fig. 1A and B). After completing the study, an independent investigator (G.J.L.O.), who was blinded to subject status, confirmed correct placement of the volumes of interest in all participants. Shimming on the volume of interest was performed using first- and second-order shims based on a calculated B_0 map. The number of scan averages was 32. A non-water suppressed spectrum was acquired from the same volume of interest, with the transmitter frequency set on the water resonance. Magnetic resonance spectra were preprocessed with a custom written script in Matlab® (version R2012a, The MathWorks, Inc., Natick, MA, USA) that yielded a weighted average of the individually-phased signals from all 32 receive channels and performed eddy current correction.

For DWS we used an adapted point resolved spectroscopy (PRESS) sequence (repetition time/echo time = 2000/120 ms, spectral width = 3 kHz, 1024 points, acquisition time ≈15 min) (Kan *et al.*, 2012). The number of scan averages was 56. Cardiac triggering was performed using the peripheral pulse unit. The transmitter frequency was set at 2.35 ppm (glutamate). Diffusion weighting was applied using bipolar gradients to reduce the effects of eddy currents on the magnetic resonance spectra (Reese *et al.*, 2003). Diffusion-weighting was applied in three quasi-orthogonal directions and spectra were obtained at two different gradient strengths (14 and 28 mT/m), with gradient duration of 38 ms and a bipolar gap of 14 ms. The diffusion time was 60 ms. This resulted in *b*-factors of $b_1 = 1634$ s/mm^2 and $b_2 = 6535$ s/mm^2 in addition to a condition without diffusion weighting ($b_0 = 0$ s/mm^2). The choice of echo time value of 120 ms lies in the range of theoretically predicted optimal values for quantification of glutamate (Snyder and Wilman, 2010). The water signal was only partially suppressed in order to acquire a residual water signal for individual phasing of each spectrum. Data were acquired as single scans and averaged off-line after channel weighing, zero- and first-order phase correction, frequency drift correction and eddy current correction (Kan *et al.*, 2012). DWS data processing was performed using a custom written script in Matlab® as described previously (Upadhyay *et al.*, 2007).

Tissue segmentation of volume of interest

To correct for intersubject differences in grey matter fraction within the MRS volume of interest in the statistical analysis, we performed tissue segmentation with FSL (version 4.1.7, FMRIB Software Library, University of Oxford). Brain extraction of the 3D T_1-weighted images was carried out using the Brain Extraction Tool (BET). Whole brain segmentation of grey matter, white matter and CSF was performed using FMRIB's Automated Segmentation Tool (FAST) segmentation. The grey matter, white matter and CSF fractions in the volume of interest where calculated based on the segmented images and volume of interest size and coordinates with a custom written script in Matlab®.

Spectral fitting

The acquired ^{1}H-MR spectra were analysed using LCModel (version 6.3-1B, Stephen Provencher, Inc., Oakville, ON, Canada), which calculates the best fit of the experimental spectrum as a linear combination of model spectra (Provencher, 2001). For the ^{1}H-MRS data we used a simulated basis set including 22 metabolites and for the DWS data, a simulated basis set including 16 metabolites. Both basis sets were generated using NMRSIM (version 4.6.a., Bruker Biospin Inc). An acquired macromolecular spectrum was included in the basis sets. The parameter DKNTMN that controls the node spacing for the spline baseline fitting was set to the commonly used value of 0.20 (Tkac *et al.*, 2009). LCModel fitting was performed over the spectral range of 0.2–4.2 ppm, appropriate for ^{1}H-MRS at 7 T.

Spectral quality assessment

Visual inspection of the spectra was performed by two investigators (R.Z. and J.W.) who were blinded for the diagnosis. Spectra showing clear artefacts, due for example, to stimulated echoes, inadequate water suppression, or poor shimming, were *a priori* excluded from further analysis. The quality of the DWS spectra was assessed in two stages. First, if the data quality was insufficient to perform individual phasing of each spectrum, the case was excluded. Second, after spectral fitting the individual DWS spectra were inspected and cases with spectra that showed clear artefacts were excluded from further analysis.

The signal-to-noise ratio (SNR) as given by LCModel is defined as the ratio of the maximum in the spectrum minus the baseline over twice the root mean square of the residuals between 0.2–4.2 ppm, and was used as a parameter to assess spectral quality. The full-width at half-maximum of *N*-acetylaspartate (NAA) is a measure of the linewidth in the spectrum. The Cramér-Rao lower bound (CRLB) of the metabolite concentrations represents the estimated standard deviation (SD) expressed as percentage of the estimated concentration; values smaller than 15% SD on average were considered reliable estimates of the metabolite concentration; if the CRLB of a given metabolite exceeded 15% SD in more than 50% of the cases that metabolite was excluded from further analysis for all cases.

Quantification of MRS and DWS data

Spectral quantification was performed using the unsuppressed water signal obtained from the same volume of interest (Gasparovic *et al.*, 2006). The relative densities of magnetic resonance visible water for grey matter, white matter and CSF were assumed to be 0.78, 0.65 and 0.97, respectively (Ernst *et al.*, 1993). The T_1 and T_2 relaxation times of water used in the calculation of the water attenuation factors for the

occipital volume of interest were: T_1(grey matter) = 2130 ms, T_1(white matter) = 1220 ms, T_1(CSF) = 4425 ms (Rooney *et al.*, 2007); T_2(grey matter) = 50 ms, T_2(white matter) = 55 ms, T_2(CSF) = 141 ms (Bartha *et al.*, 2002). The water attenuation was calculated for every subject separately based on the segmentation results of the volume of interest. As data were acquired with a long repetition time, partial saturation due to T_1 relaxation of the metabolites was not taken into account. The T_2 values of glutamate [T_2(Glu) = 93 ms] and other metabolites were taken from literature data (Marjanska *et al.*, 2012). The apparent diffusion coefficient (ADC) values were calculated for each direction from the DWS spectra using a mono-exponential fit; the final ADC was then calculated by averaging the ADC values for the three directions.

Migraine symptoms and visual sensitivity measures

All participants with migraine kept a headache diary covering the 7 days before and after examination and completed a questionnaire about their migraine characteristics, the Migraine Disability Assessment Scale (MIDAS) (Stewart *et al.*, 2001), and the Headache Impact Test (HIT-6) (Kosinski *et al.*, 2003). To assess any potential relation between glutamate concentrations in the visual cortex and visual sensitivity, all participants also completed a 9-item validated Visual Sensitivity Questionnaire (VSQ), which quantifies interictal sensitivity to light and visual patterns (Perenboom *et al.*, 2016). In addition, all participants underwent a Pattern Glare Test immediately after the scan, which quantifies visual perceptual distortions and visual illusions on viewing three striped patterns with different spatial frequencies (Evans and Stevenson, 2008). For the Pattern Glare Test, a General Illusion Index was calculated, which is the average number of distortions over the three different striped patterns (Shepherd *et al.*, 2013).

Statistical analysis

Power calculations for estimating the required number of subjects were based on estimates of glutamate concentration from literature (Marjanska *et al.*, 2012). To detect 10% difference in glutamate concentration with a power of 80% in a two-sided *t*-test, at least 20 participants per group were needed. Statistical analysis was performed with SPSS (version 20.0, IBM SPSS Statistics for Windows, Armonk, NY: IBM Corp). Data were checked for normality of distribution by means of a Kolmogorov-Smirnov test and equality of variances with Levene's test. To test for differences between groups in clinical and demographic characteristics we used a Chi-square test for proportions, a Kruskal-Wallis test or a Mann-Whitney test to test for differences in non-normal distributed continues variables, and a one-way ANOVA for normally distributed variables. The primary analysis assessed differences in metabolite concentrations between (only) interictal migraineurs with and without aura, versus healthy controls, using an analysis of covariance (ANCOVA) with the grey matter fraction within the volume of interest as covariate. *Post hoc* pairwise comparisons were done by means of ANCOVA of the adjusted means with grey matter as covariate and adjusted for multiple comparisons using Bonferroni correction. Differences in apparent diffusion coefficient were tested with a one-way ANOVA. To assess whether the glutamate concentrations in the visual cortex are related to the time point in the migraine cycle, we used Pearson's correlation to test for a relation between the glutamate concentration and number of days before the next attack. In addition, we tested for differences in glutamate concentration between interictal and preictal migraineurs with an ANCOVA with the grey matter fraction within the volume of interest as covariate. To increase the statistical power we pooled both interictal and preictal migraineurs in a secondary analysis. To assess the potential relation between the glutamate concentration in the visual cortex and the clinical measures of visual sensitivity, i.e. sum score of the VSQ and the General Illusion Index of the pattern glare test, we used Pearson's correlation. *P*-values < 0.05 were considered significant.

Results

Spectral quality assessment

^{1}H-MRS and DWS data were obtained from 36 participants with migraine without aura (27 interictal and nine preictal), 27 with migraine with aura (23 interictal and four preictal) and 27 non-headache control subjects (Fig. 2). An example of an ^{1}H-MR spectrum and diffusion weighted spectra acquired from a single subject are shown in Fig. 1C and D, respectively. ^{1}H-MR spectra from three subjects were excluded from the analysis because of insufficient spectral quality and clearly visible artefacts (Fig. 2). Five DWS datasets were not fully acquired due to time constraints and therefore the data were excluded. The DWS data quality from six subjects was insufficient to perform individual phasing of the spectra; in addition, the spectral quality was insufficient or showed clear artefacts in six other subjects; these 12 subjects were therefore excluded from further analysis (Fig. 2). In summary, after exclusion of the above datasets, there were for the primary analysis 27 MRS and 23 DWS scans available from participants with migraine without aura, 23 MRS and 22 DWS scans from participants with migraine with aura and 24 MRS and 18 DWS from non-headache controls. The exclusion of these datasets did not cause any between-group imbalance in age, gender or migraine characteristics.

The SNR of the ^{1}H-MR spectra that passed quality control was 41.6 ± 9.6 and the full-width at half-maximum of the NAA peak was 13.4 ± 2.9 Hz. The CRLBs for glutamate and glutamine were 2.8 ± 1.0 and 8.8 ± 2.7, respectively. These values imply that LCModel was able to perform accurate fitting of glutamate and glutamine individually. The SNR of the diffusion weighted spectra was 13.6 ± 3.0 in the non-diffusion weighted condition b_0 and decreased to 8.7 ± 2.5 in the highest diffusion-weighted condition b_2. The full-width at half-maximum was 11.2 ± 2.2 in condition b_0 and 12.3 ± 2.7 in condition b_2 (no significant difference). The CRLB of glutamate increased from 6.3 ± 1.7 in condition b_0 to 9.4 ± 3.8 in the diffusion-weighted condition b_2, which is well within the conservative quality limit of CRLB less than 15% that

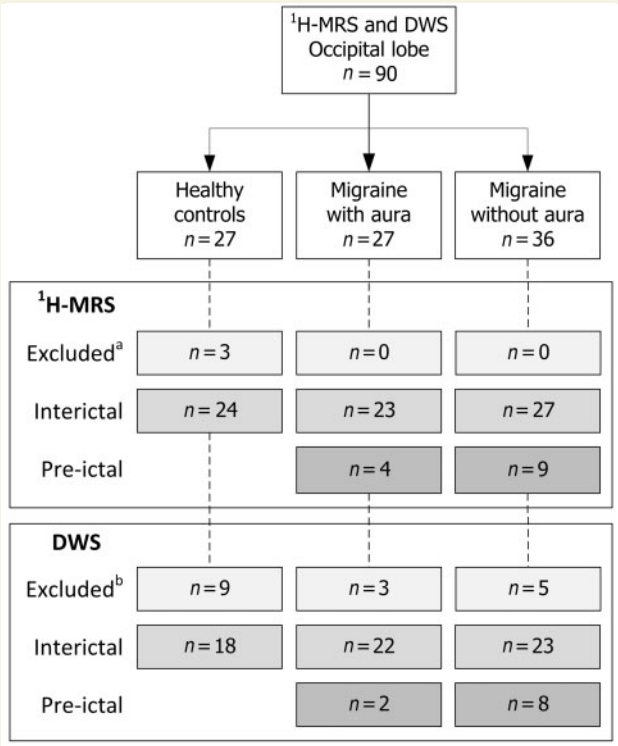

Figure 2 Flowchart of subject stratification for ^{1}H-MRS and DWS analysis. Interictal = attack free for 72 h before and 48 h after investigation; preictal = attack free for 72 h before investigation but with a migraine attack within 48 h after investigation.
[a]Subjects excluded because of insufficient spectral quality and clear artefacts.
[b]Subjects excluded because DWS data were not (fully) acquired, DWS data quality was considered insufficient after preprocessing, or because of insufficient spectral quality and clear artefacts.

we defined at the start of the study. There was full agreement between the original placements and the placements by an independent blinded investigator (G.J.L.O.) after the study had been completed in all but four cases (two controls, one migraine with aura, and one migraine without aura). The differences in volume of interest placement in these four cases, however, were only minor (1–2 mm). The overlap in volume of the individual volume of interest placements was still 95%.

Clinical characteristics

The demographic and clinical characteristics of the participants included in the primary analysis are shown in Table 1 and of those included in the secondary analysis, where interictal and preictal migraineurs were pooled, are shown in Table 2. Participants with migraine with and without aura did not differ in general migraine characteristics and migraine-related disability. Participants with migraine with aura experienced visual aura symptoms in 83.7% of their attacks. Participants with migraine had higher visual sensitivity as measured with the VSQ ($P < 0.001$) and experienced more visual illusions when looking at different striped patterns during the Pattern Glare Test ($P = 0.001$) than non-headache controls. For both tests, there were no differences between participants with migraine with or without aura.

Glutamate concentrations and diffusion

Participants with migraine with or without aura had slightly higher grey matter fraction and lower CSF fractions in the volume of interest compared to controls (Table 1). Glutamate concentrations correlated with the grey matter fractions in the volume of interest (Pearson's r = 0.51, $P < 0.001$); the grey matter fraction was therefore included as a covariate.

In the primary analysis, glutamate levels were different when comparing the three primary study groups, i.e. interictal participants with migraine with aura ($n = 23$) or without aura ($n = 27$) and controls [$n = 24$, $F(2,70) = 3.20$; $P = 0.047$, Table 3 and Fig. 3A]. In a *post hoc* analysis, glutamate levels were 6% higher in migraine without aura compared to controls ($P = 0.042$); however, no differences were found between migraine with aura and controls ($P = 0.72$), nor between migraine with and without aura ($P = 0.53$).

The glutamate concentrations did not significantly differ between migraine participants who were measured interictally and of those who were measured preictal [$F(1,84) = 1.96$; $P = 0.16$]. There was no correlation between the number of days before a next attack and the glutamate concentration (Pearson's r = −0.18; $P = 0.29$; Fig. 4). We pooled the inter- and preictal concentrations in a secondary analysis (Table 3 and Fig. 3B). The intergroup differences across pooled migraine with aura ($n = 27$), pooled migraine without aura ($n = 36$) and controls ($n = 24$) were similar, but statistically considerably stronger, compared to those observed in the primary analysis only including interictal concentrations. Thus, the glutamate concentration differed across groups [$F(2,83) = 1.78$; $P = 0.004$] and, in migraine without aura, was higher compared to controls ($P = 0.006$) and near-significantly higher compared to migraine with aura ($P = 0.051$).

No differences were found for the ADC of glutamate in both the primary and secondary analyses, nor for the concentration and ADC of the other metabolites (Table 3).

Clinical parameters and glutamate

The glutamate concentration in the visual cortex did not correlate with the self-reported visual sensitivity (sumscore on the VSQ) (Pearson's r = 0.17, $P = 0.126$) or with the General Illusion Index from the Pattern Glare Test (Pearson's r = 0.01, $P = 0.918$) when looking at all subjects, nor when looking at subgroups of interictal or pooled interictal and preictal migraineurs separately.

Table 1 Clinical and demographic characteristics of healthy controls and interictal migraineurs ($n = 74$)

	Healthy controls, $n = 24$	Migraine without aura, $n = 27$	Migraine with aura, $n = 23$	*P*-value
General				
Female (*n*, %)	12 (50.0)	14 (51.9)	11 (47.8)	0.961[a]
Age	34.8 ± 8.7	35.1 ± 8.2	35.0 ± 9.3	0.992[b]
BMI	23.3 ± 2.5	22.6 ± 1.8	23.9 ± 2.6	0.325[b]
Smoking (*n*, %)	2 (8.3)	5 (18.5)	3 (13.0)	0.567[a]
Migraine characteristics				
Age at onset	-	14.2 ± 7.7	14.4 ± 8.1	0.938[c]
Attack frequency (attack/month)	-	2.8 ± 2.5	2.3 ± 1.4	0.911[c]
Attack duration (h)	-	36.6 ± 26.8	27.3 ± 21.0	0.235[c]
Headache days (days/month)	-	7.4 ± 5.0	5.4 ± 4.7	0.051[c]
Aura (% of attacks)	-	-	83.7 ± 22.6	-
Migraine-related disability				
MIDAS	-	16.7 ± 15.2	15.1 ± 13.9	0.733[c]
HIT-6	-	62.6 ± 4.4	62.8 ± 6.3	0.614[c]
Visual sensitivity				
VSQ (sumscore)	3.2 ± 2.6	8.4 ± 4.8	12.4 ± 7.6	**<0.001**[b]
Pattern glare (GII)	2.7 ± 2.3	5.1 ± 2.1	5.1 ± 1.7	**0.001**[b]
Tissue segmentation of VOI				
Grey matter fraction	0.62 ± 0.05	0.65 ± 0.03	0.65 ± 0.04	**0.020**[d]
White matter fraction	0.28 ± 0.04	0.28 ± 0.04	0.28 ± 0.04	0.871[d]
CSF fraction	0.10 ± 0.06	0.07 ± 0.02	0.07 ± 0.04	0.059[d]

BMI = body mass index; MIDAS = migraine disability assessment scale; HIT-6 = Headache Impact Test; VSQ = Visual Sensitivity Questionnaire; VOI = volume of interest.
Values are expressed as mean ± SD.
[a]Chi-square test.
[b]Kruskal-Wallis test.
[c]Mann-Whitney test.
[d]One-way ANOVA.
P-values < 0.05 are depicted in bold.

Table 2 Clinical and demographic characteristics of healthy controls and inter- plus pre-ictal migraineurs ($n = 87$)

	Healthy controls, $n = 24$	Migraine without aura, $n = 36$	Migraine with aura, $n = 27$	*P*-value
General				
Female (*n*, %)	12 (50.0)	19 (52.8)	14 (51.9)	0.978[a]
Age	34.8 ± 8.7	35.4 ± 8.2	35.3 ± 9.2	0.984[b]
BMI	23.3 ± 2.5	22.8 ± 1.8	23.5 ± 2.7	0.818[b]
Smoking (*n*, %)	2 (8.3)	5 (13.9)	3 (11.1)	0.802[a]
Migraine characteristics				
Age at onset	-	14.1 ± 7.1	14.4 ± 7.6	0.906[c]
Attack frequency (attack/month)	-	3.0 ± 2.3	2.3 ± 1.4	0.415[c]
Attack duration (h)	-	32.1 ± 26.5	28.7 ± 22.1	0.752[c]
Headache days (days/month)	-	7.1 ± 4.6	5.6 ± 4.5	0.092[c]
Aura (% of attacks)	-	-	83.9 ± 21.8	-
Migraine related disability				
MIDAS	-	16.4 ± 15.9	18.3 ± 19.5	0.701[c]
HIT-6	-	62.7 ± 4.3	63.2 ± 5.9	0.443[c]
Visual sensitivity				
VSQ (sum score)	3.2 ± 2.6	8.7 ± 5.0	12.3 ± 7.2	**<0.001**[b]
Pattern glare (GII)	2.7 ± 2.3	5.1 ± 2.2	4.8 ± 1.8	**0.001**[b]
Tissue segmentation of VOI				
Grey matter fraction	0.62 ± 0.05	0.65 ± 0.04	0.65 ± 0.05	**0.013**[d]
White matter fraction	0.28 ± 0.04	0.28 ± 0.04	0.28 ± 0.04	0.831[d]
CSF fraction	0.10 ± 0.06	0.07 ± 0.03	0.07 ± 0.04	**0.036**[d]

MIDAS = migraine disability assessment scale; GII = General Illusion Index; HIT-6 = Headache Impact Test; VOI = volume of interest; VSQ = Visual Sensitivity Questionnaire.
Values are expressed as mean ± SD. Statistically significant *P*-values are highlighted in bold.
[a]Chi-square test.
[b]Kruskal-Wallis test.
[c]Mann-Whitney test.
[d]One-way ANOVA.

Table 3 Average metabolite concentrations and apparent diffusion coefficients in the visual cortex

	Healthy controls	Interictal migraine patients		*P*-value	Interictal plus preictal migraine patients		*P*-value
		Migraine without aura	Migraine with aura		Migraine without aura	Migraine with aura	
Concentration (mmol/l)[a]							
Primary outcome	*n* = 24	*n* = 27	*n* = 23		*n* = 36	*n* = 27	
Glu	6.40 ± 0.78	7.02 ± 0.50	6.77 ± 0.47	**0.047**	7.07 ± 0.56	6.76 ± 0.51	**0.004**
Secondary outcomes							
Gln	2.15 ± 0.42	2.40 ± 0.38	2.26 ± 0.51	0.194	2.43 ± 0.39	2.27 ± 0.48	0.109
Glu + Gln	8.55 ± 1.12	9.42 ± 0.76	9.03 ± 0.81	0.051	9.50 ± 0.86	9.03 ± 0.83	**0.008**
tNAA	10.29 ± 0.87	10.85 ± 0.52	10.69 ± 0.60	0.180	10.82 ± 0.56	10.71 ± 0.64	0.164
tCr	7.24 ± 0.69	7.72 ± 0.58	7.54 ± 0.45	0.101	7.69 ± 0.58	7.60 ± 0.49	0.105
Ins	4.08 ± 0.46	4.19 ± 0.38	4.30 ± 0.61	0.422	4.22 ± 0.39	4.30 ± 0.65	0.503
tCho	1.32 ± 0.26	1.35 ± 0.16	1.25 ± 0.17	0.220	1.35 ± 0.15	1.26 ± 0.19	0.179
PE	3.41 ± 0.98	3.58 ± 0.54	3.42 ± 0.62	0.782	3.54 ± 0.58	3.48 ± 0.64	0.877
Asp	3.73 ± 1.10	3.73 ± 0.65	3.58 ± 0.67	0.762	3.73 ± 0.65	3.61 ± 0.66	0.820
ADC ($\mu m^2/s$)[b]							
Primary outcome	*n* = 18	*n* = 23	*n* = 22		*n* = 31	*n* = 24	
Glu	0.106 ± 0.018	0.108 ± 0.025	0.120 ± 0.027	0.129	0.110 ± 0.026	0.110 ± 0.027	0.204
Secondary outcomes							
Glu + Gln	0.106 ± 0.018	0.107 ± 0.025	0.118 ± 0.028	0.194	0.109 ± 0.027	0.117 ± 0.028	0.317
tNAA	0.107 ± 0.022	0.114 ± 0.026	0.127 ± 0.028	0.057	0.115 ± 0.026	0.124 ± 0.028	0.109
tCr	0.110 ± 0.015	0.117 ± 0.020	0.120 ± 0.019	0.247	0.118 ± 0.019	0.120 ± 0.019	0.220
tCho	0.099 ± 0.014	0.104 ± 0.018	0.108 ± 0.020	0.287	0.104 ± 0.018	0.109 ± 0.019	0.233

Interictal = no migraine attack in the 3 days before and the 2 days after examination; preictal = no migraine attack in the 3 days before examination but experiencing an attack within the 2 days following examination. Values are expressed as actual means ± SDs. Statistically significant *P*-values are highlighted in bold.
Glu = glutamate; Gln = glutamine; tCr = total creatine; Ins = myo-inositol; tCho = total choline; PE = phosphorylethanolamine; Asp = aspartate.
[a]*P*-values from one-way ANCOVA between groups with grey matter fraction as covariate.
[b]*P*-values from a one-way ANOVA.

Discussion

In the present study, we used ^{1}H-MRS and DWS at 7 T to measure interictal levels of glutamate and glutamine in the visual cortex of migraine patients. Glutamate levels were increased in participants with migraine without aura but not in those with migraine with aura. Glutamine concentrations and intracellular mobility of glutamate were normal.

Although the main aim of the study was to assess baseline cortical glutamate and glutamine concentrations outside attacks, 13 migraine participants were accidentally investigated within 2 days before an attack. These preictal concentrations did not, however, differ from interictal concentrations, suggesting that increased cortical glutamate is a permanent and relatively stable feature of patients with migraine without aura.

^{1}H-MRS and DWS primarily measure intraneuronal glutamate, which accounts for ~80% of glutamate present in the brain (Erecinska and Silver, 1990; Danbolt, 2001). We believe our results indicate increased intracellular levels of glutamate rather than increased number of glutamatergic neurons as we failed to find any difference for the neuronal marker total NAA (Moffett *et al.*, 2007) (Table 2). Moreover, we also corrected for grey matter content of the volume of interest, which primarily consists of neurons.

Glutamate is distributed over different intracellular compartments, mainly the cytosol, cell organelles, and synaptic vesicles (Danbolt, 2001; Zhou and Danbolt, 2014). The mobility of glutamate within and between these compartments is influenced by the size of the compartments, obstructions in and between the compartments, and properties of the cytosol, such as viscosity (Nicolay *et al.*, 2001). The diffusion properties of metabolites indirectly provide information on the compartmentalization of metabolites within tissue compartments, on changes in cellular microstructure caused by disease or other factors, and on changes in cytosol properties, e.g. viscosity, tortuosity, and macromolecular crowding (Nicolay *et al.*, 2001). Although pathology-driven changes in compartmentalization of glutamate have been implicated in certain neurological diseases (Beal, 1992; Coyle and Puttfarcken, 1993; Valette *et al.*, 2005), the normal intracellular mobility of glutamate in our study makes such mechanisms in migraine unlikely.

A major strength of our study is that we used ^{1}H-MRS at high magnetic field strength (7 T) enabling accurate and separate assessment of glutamate and glutamine as confirmed by the low CRLBs of glutamate (2.8 ± 0.95) and glutamine (8.8 ± 2.7). This is a major advantage as both

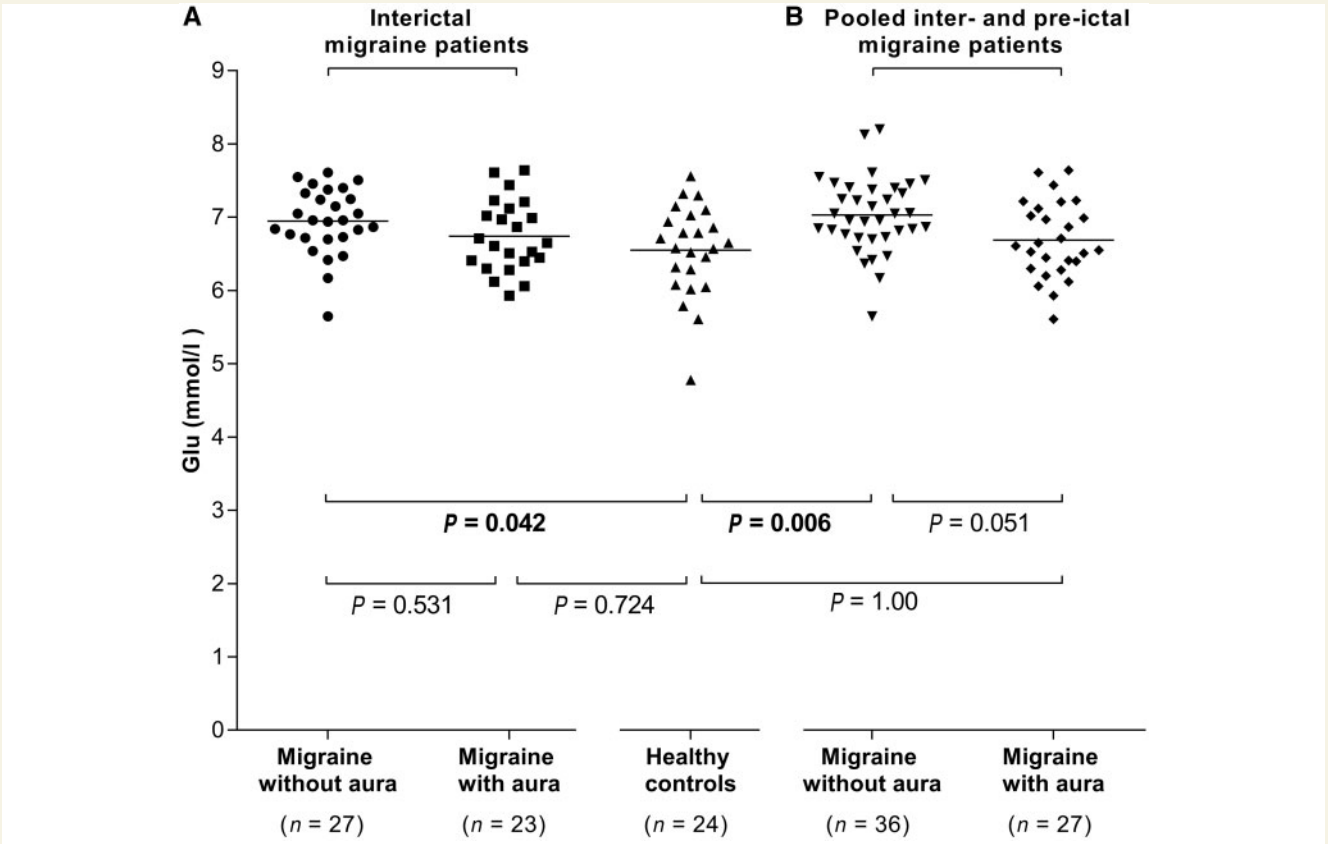

Figure 3 Glutamate (Glu) concentrations adjusted for grey matter fraction. (**A**) Primary analysis for group difference between the concentrations in the control group and the interictal values in migraineurs with or without aura [$F(2,70) = 3.20$; $P = 0.047$]. (**B**) Secondary analysis for group differences between the concentrations in the control group and the pooled interictal and preictal values of migraineurs with or without aura [$F(2,83) = 1.78$; $P = 0.004$]. *P*-values from *post hoc* tests with Bonferroni correction after one-way ANCOVA between groups with grey matter fraction as covariate.

amino acids, although metabolically intimately related via the glutamate glutamine cycle, have very different functions. While glutamate acts as the main excitatory neurotransmitter, glutamine is essentially inactive and primarily functions as a precursor to glutamate (Danbolt, 2001).

Previous ^{1}H-MRS studies in migraine have used techniques that were not optimized to accurately differentiate between glutamate and glutamine and consequently could only report the combined signal of glutamate and glutamine (Glx, in some studies simply reported as Glu) (Dichgans *et al.*, 2005; Prescot *et al.*, 2009; Siniatchkin *et al.*, 2012; González *et al.*, 2013; Zielman *et al.*, 2014; Bridge *et al.*, 2015; Arngrim *et al.*, 2016). One study reported an increased Glx/creatine ratio in the occipital lobe of 10 patients with migraine with aura (Siniatchkin *et al.*, 2012), while five other studies failed to find any difference in Glx (Dichgans *et al.*, 2005; González *et al.*, 2013; Zielman *et al.*, 2014; Bridge *et al.*, 2015; Arngrim *et al.*, 2016). In one study the glutamate/glutamine ratio was increased in patients with migraine with or without aura (González *et al.*, 2013) potentially suggesting a higher neuron-to-astrocyte ratio as glutamate is mainly present in neurons and glutamine in astrocytes. However, the CRLB for glutamine in that study was high (34.1% ± 19.2), indicating considerable margin of measurement error. In our study, the glutamate/glutamine ratio in the occipital lobe of migraine patients was normal. In a recent study using ^{1}H-MRS, primarily to measure lactate changes after hypoxia, cortical glutamate levels did not differ at baseline between patients with migraine and healthy controls (Arngrim *et al.*, 2016). However, the 3 T MRS technique in this study was not optimized to separate glutamate from glutamine. As a consequence, Glx (glutamate and glutamine together) was probably estimated. Moreover, no information was provided on the quality of the spectra LCModel fits. In the present study, we do not report lactate concentrations because lactate could not be fitted reliably. For robust detection of lactate, dedicated MRS editing sequences are needed (Arteaga de Castro *et al.*, 2013; Wijnen *et al.*, 2015). These were, however, not operational at our department at the time of the present study. Moreover, adding such sequences would significantly have increased measurement time and thus burden for the participants.

As summarized in Table 3, we did not find differences between migraineurs and controls for the cerebral concentrations of total NAA, total creatine (tCr), myo-inositol

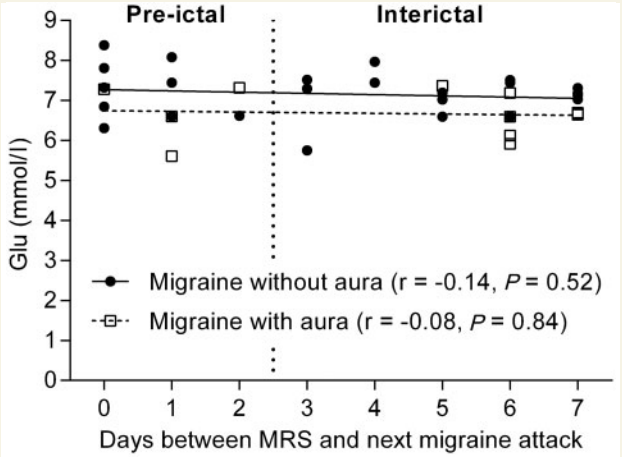

Figure 4 Relation between days until next attack and glutamate concentration. All subjects with attack within 7 days after study day (n = 37).

(Ins), total choline (tCho), phosphoroylethanolamine (PE) or Asp. Other studies using ^{1}H-MRS to measure brain metabolism in migraine have yielded disparate results (Reyngoudt *et al.*, 2012; Younis *et al.*, 2017). This has most likely been due to a combination of a variety of methodological differences including heterogeneous migraine populations, different timings of the measurement (ictal versus interictal), and differences in MRS techniques, field strengths, signal processing, and quantification strategies. In a recent study, using 2D ^{1}H-MRS, no differences were found between interictal metabolite concentrations in the anterior cingulate cortex of healthy controls and patients with migraine with or without aura when applying univariate statistics (Becerra *et al.*, 2016). However, when applying a quadratic discriminant analysis model, they found a complex of metabolite ratios (Asp/Cre, NAA/Cre, and Gln/Cre), which could discriminate migraineurs from controls. The exact direct of the changes, however, was not made clear from the study results.

Increased glutamatergic activity has long been implicated in migraine pathophysiology through various putative mechanisms (Ferrari *et al.*, 1990). A major hypothesis has been that increased extracellular glutamate within the synaptic cleft would lead to cortical hyperexcitability and enhanced susceptibility to CSD (Tottene *et al.*, 2009; Pietrobon and Moskowitz, 2014; Ferrari *et al.*, 2015). As CSD is considered the underlying mechanism for migraine aura, one would have expected to find increased cortical glutamate in migraine with aura and possibly also migraine without aura (Bolay, 2012; Karatas *et al.*, 2013; Ferrari *et al.*, 2015). Instead we found increased glutamate levels only in migraine without aura but not in migraine with aura.

Of relevance here is to realise that with the currently available measurement techniques one can only measure the total of the extracellular and the 10 000-fold higher intracellular glutamate (Erecinska and Silver, 1990). At present, there is no method to distinguish these two glutamate compartments in humans, not even with high field ^{1}H-MRS and DWS. We therefore cannot confirm nor refute whether synaptic (extracellular) glutamate is changed in migraine with or without aura.

A second possible role for glutamate in migraine would be as part of the cortical glutamatergic excitation/GABAergic inhibition balance. Unfortunately, in our study, GABA could not be fitted reliably because we didn't use dedicated MRS editing sequences, which are required for robust detection of GABA (Arteaga de Castro *et al.*, 2013). In a recent study, in which a dedicated editing sequence for GABA was applied, increased levels of GABA + (GABA plus substantial macromolecule contamination) were observed in the posterior cingulate cortex of patients with migraine with and without aura (Aguila *et al.*, 2015). No subanalyses were done separately for patients with migraine with and those without aura. GABA levels in that study positively correlated with pain and central sensitization scores (Aguila *et al.*, 2016). In our study, we didn't observe any correlation between clinical parameters such as sensitivity to light and patterns and occipital glutamate concentrations.

Increase of glutamate might also be related to the role of glutamate in the energy metabolism of the brain. Glutamate is a central compound in cellular metabolism since its carbon skeleton is an input into several anabolic and catabolic pathways. The formation and degradation of glutamate is also part of the general energy metabolism of the brain, as it leaves and enters the tricarboxylic acid (TCA) cycle at the level of α-ketoglutarate. Glutamate can re-enter the TCA cycle as an alternative oxidizable substrate and as such can serve as an important energy reserve (Danbolt, 2001; McKenna, 2013). As glutamate is such a key component of intermediary metabolism, the results of our study might also reflect changes in intracellular metabolism or an increase in alternative energy reserves. This would be well in line with previous observations with ^{31}P-MRS showing reduced interictal levels of high energy phosphates such as adenosine triphosphate (ATP) and phosphocreatine (PCr) in migraine with and without aura. These observations would suggest disrupted energy metabolism and reduced energy reserve as a key mechanism in migraine (Reyngoudt *et al.*, 2012).

Maintaining low extracellular glutamate concentrations via glutamate uptake by neurons, astrocytes, and the blood–brain barrier is also highly energy-dependent (Zhou and Danbolt, 2014). One could speculate that a slight impairment in glutamate uptake due to smaller energy reserves may lead to higher extracellular levels of glutamate (Danbolt, 2001), which in turn might increase cortical excitability and migraine susceptibility. The connection between glutamate as neurotransmitter—which compartmentalization is highly energy-dependent—and glutamate as an intermediate in energy metabolism should be investigated in more detail in migraine patients. This could be done, e.g. by simultaneously

using ^{1}H-MRS and ^{31}P-MRS techniques, or by using ^{13}C-MRS to study neuro-energetics and neurotransmitter cycling in the brain (Rothman *et al.*, 2011). Future studies in migraine might also use functional MRS at 7 T to investigate neurotransmitter dynamics following visual stimulation. Recent functional MRS studies at 7 T found a small but marked increase of glutamate (2–3% increase) and lactate (19–23%) concentrations in the occipital lobe after visual stimulation (Mangia *et al.*, 2007; Lin *et al.*, 2012; Schaller *et al.*, 2013). It will be interesting to investigate whether differences in cortical excitability, e.g. increased evoked potentials after visual stimulation, is associated with functional changes in brain metabolites.

As well as several strengths, such as: (i) use of 7 T ^{1}H-MRS and DWS, providing information on glutamate concentration and mobility; (ii) strict inclusion criteria to clearly distinguish participants with migraine with aura from those without aura; and (iii) inclusion of interictal and preictal migraineurs, our study also has some limitations. Although our study population as a whole is larger than those investigated in other MRS studies, the subgroups are still small and might thus be underpowered to detect differences e.g. between migraine with and without aura or between interictal and preictal migraineurs. Moreover, we could only measure the ADC of glutamate but not of glutamine because we selected an echo time of 120 ms, which falls in the range of theoretically predicted optimal values for quantification of glutamate and was chosen based on the need to accommodate the diffusion weighting gradients. The investigator who performed the measurements was not blinded for subject status. To avoid possible bias in volume of interest placement, the sizes of the volumes of interest were fixed and clear anatomical landmarks were used for placement of the volumes of interest. In addition, an independent investigator (G.J.L.O.) who was blinded to subject status, verified correct placement of the volumes of interest in all participants. As the differences were only minimal and equally affected all three subgroups, a systematic (biased) error in volume of interest placement seems very unlikely. Finally, for scientific and methodological reasons we focused on the visual cortex as region of primary interest. We, therefore, cannot extrapolate our findings to other brain regions.

In conclusion, glutamate levels are increased in the visual cortex of patients with migraine without aura, strengthening the pathophysiological link of this amino acid with migraine, either through its neuroexcitatory effects, its role in energy metabolism, or a combination of both.

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Conflicts of interest

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