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Transcranial Magnetic Stimulation Potentiates Glutamatergic Neurotransmission in Depressed Adolescents

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Abstract

Abnormalities in glutamate neurotransmission may have a role in the pathophysiology of adolescent depression. The present pilot study examined changes in cortical glutamine/glutamate ratios in depressed adolescents receiving high-frequency repetitive transcranial magnetic stimulation. Ten adolescents with treatment-refractory major depressive disorder received up to 30 sessions of 10-Hz repetitive transcranial magnetic stimulation at 120% motor threshold with 3,000 pulses per session applied to the left dorsolateral prefrontal cortex. Baseline, posttreatment, and 6-

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Contributions

Author contributions: All authors contributed to the conception and design of the study. Drs. Croarkin, Wall, Murphy, and Port collected and assembled the data. Drs. Croarkin, Nakonezny, Frye and Port analyzed and interpreted the data. All authors contributed to writing the manuscript and gave final approval of the manuscript. Dr. Croarkin had full access to all data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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month follow-up proton magnetic resonance spectroscopy scans of the anterior cingulate cortex and left dorsolateral prefrontal cortex were collected at 3T with 8-cm³ voxels. Glutamate metabolites were quantified with 2 distinct proton magnetic resonance spectroscopy sequences in each brain region. After repetitive transcranial magnetic stimulation and at 6 months of follow-up, glutamine/glutamate ratios increased in the anterior cingulate cortex and left dorsolateral prefrontal cortex with both measurements. The increase in the glutamine/glutamate ratio reached statistical significance with the TE-optimized PRESS sequence in the anterior cingulate cortex. Glutamine/glutamate ratios increased in conjunction with depressive symptom improvement. This reached statistical significance with the TE-optimized PRESS sequence in the left dorsolateral prefrontal cortex. High-frequency repetitive transcranial magnetic stimulation applied to the left dorsolateral prefrontal cortex may modulate glutamate neurochemistry in depressed adolescents.

Keywords

adolescent depression; glutamate; glutamine; proton magnetic resonance spectroscopy; repetitive transcranial magnetic stimulation

1. Introduction

Major depressive disorder (MDD) frequently presents in adolescence and is often recalcitrant to treatment (Brent, 2009) which leads to substantial morbidity, mortality, and a societal financial burden (Blazer et al., 1994; Greenberg et al., 2015). Suicide is a leading cause of death in adolescents and a stark reminder that the current mechanistic understanding of depression is underdeveloped (Vitiello et al., 2011). Unfortunately, antidepressant medications, cognitive-behavioral therapy, and combined treatment are either ineffective or have minimal durability for most depressed adolescents (March et al., 2009).

Noninvasive brain stimulation technologies such as repetitive transcranial magnetic stimulation (rTMS) may have promise as enduring therapeutic interventions in young people (Donaldson et al., 2014). Prior research has shown that rTMS applied to the left dorsolateral prefrontal cortex (L-DLPFC) is a safe and effective treatment for MDD in adults who fail to benefit from antidepressant medications (O'Reardon et al., 2007; George et al., 2010). Initial open-label studies of rTMS for MDD in adolescents suggest that it may be effective and well-tolerated in younger people, as well (Donaldson et al., 2014). Although rTMS treatment has US Food and Drug Administration clearance for adults, little is known about its mechanism of action and target engagement, especially in adolescents. Further research focused on understanding the underlying pathophysiology of MDD and how rTMS changes a patient's underlying neurophysiology would facilitate precision medicine approaches to brain stimulation treatments.

Glutamate (Glu) is the primary excitatory neurotransmitter, with roles in neurogenesis, synaptogenesis, neuronal migration, cognition, learning, and memory. Following release into the synaptic cleft, Glu is taken up by adjacent astrocytes and converted to glutamine (Gln) which is then transported back to the neuron. The glutamate-glutamine neurotransmitter recycling system is essential for normal neurotransmission (Yuksel and Ongur 2010) and drives a large fraction of cerebral oxidative metabolism.

Prior research implicates dysregulated glutamatergic neurotransmission in mood disorders (Krystal et al., 2002; Sanacora et al., 2012) and suggests that rTMS may modulate glutamatergic circuitry (Michael et al., 2003). Initial preclinical (Yue et al., 2009) and clinical work (Michael et al., 2003) suggests that multiple sessions of rTMS increase cortical Glu concentrations in the brain.

Many previous proton magnetic resonance spectroscopy (^1H -MRS) studies have examined Glu+Gln (so-called Glx) levels in psychiatric disorders, while contemporary studies at higher field strength (3T and above) have been able to separately measure Glu and Gln. While it is relatively easy to quantify brain Glu (in part due to its higher concentration of approximately 10 mM), accurate measurement of brain Gln remains difficult due to its relatively low brain concentration (estimated at 2–4 mM) and large spectral overlap with other brain metabolites (Hancu and Port 2011). To mitigate this measurement difficulty and improve sensitivity for detecting glutamate-glutamine cycle dysfunction, recently investigators have used the Gln/Glu ratio (Brennan et al., 2010; Ongur et al., 2011) or the Glu/Gln ratio (Hermann, 2012) for detecting neurotransmission abnormalities in patients with psychiatric disorders.

In this study, we assumed that depressed adolescents have glutamate-glutamine cycle dysfunction. The study objective was to examine changes in Gln/Glu ratios in the anterior cingulate cortex and left dorsolateral prefrontal cortex of depressed adolescents receiving high-frequency rTMS. We hypothesized that the Gln/Glu ratio would increase over time as Gln levels would increase and Glu levels would stay the same or decrease following treatment (Yuksel and Ongur 2010). We also hypothesized that this change would relate to symptom improvement.

2. Methods

2.1. Participants

Participants were recruited and enrolled from the Mayo Clinic Mood Disorders Clinic for this prospective, open-label study of rTMS. The patient group consisted of 10 adolescents aged 13 to 17 years with treatment-refractory MDD. Parents provided informed consent and adolescents provided informed assent. Adolescents were evaluated and monitored by a child and adolescent psychiatrist for the duration of the study. Baseline assessments to determine eligibility included a semistructured diagnostic interview, the Schedule for Affective Disorders and Schizophrenia for School-Age Children-Present and Lifetime Version (Geller et al., 2001), review of Diagnostic and Statistical Manual of Mental Disorders Text Revision, Fourth Edition criteria for MDD (American Psychiatric Association, 2000), and a Children's Depression Rating Scale-Revised (CDRS-R) assessment (Poznanski et al., 1984). A CDRS-R symptom severity score of 40 or greater was required for inclusion. All participants had at least 1 prior failed trial of antidepressant medications in the current depressive episode on the basis of Antidepressant Treatment History Form (Sackeim, 2001) standards. Participants received a stable dose of an antidepressant medication for the duration of the study. Participants with secondary comorbid conditions such as anxiety or attention deficit/hyperactivity disorder were eligible for enrollment. Patients with a diagnosis of schizophrenia, schizoaffective disorder, bipolar disorder, substance abuse,

substance dependence, somatoform disorder, obsessive-compulsive disorder, posttraumatic stress disorder, eating disorder, intellectual disability, or pervasive developmental disorders were excluded. Study procedures were reviewed and approved by the Mayo Clinic Institutional Review Board before enrollment of the first participant.

2.2. rTMS technique

The *abductor pollicis brevis* site on the motor cortex was identified with standard procedures, as published elsewhere (O'Reardon et al., 2007; George et al., 2010). The resting motor threshold was determined at baseline and after every 10 sessions of rTMS for dosing. The L-DLPFC treatment site was identified with magnetic resonance imaging (MRI) under the supervision of a neuroradiologist (J.D.P.). Treatment sessions were delivered with a Neuronetic Neurostar Therapy System (Neuronetics Inc.). Stimulation was applied to the L-DLPFC at 120% motor threshold and 10 Hz frequency. Stimulus trains were 4 seconds and intertrain intervals were 26 seconds, with 3,000 pulses delivered during every session. Participants were offered the opportunity to complete up to 30 treatment sessions over 6 to 8 weeks. Posttreatment ^1H -MRS was performed after completion of or upon exit from the treatment portion of the study. As a result, posttreatment ^1H -MRS was performed 6 weeks after baseline in 4 participants and 8 weeks after baseline in 6 participants.

2.3. MRI and ^1H -MRS acquisition

Total scan time for MRI was 1 hour with a GE 3T Discovery 750 MRI scanner with 22.1 software and an 8-channel head coil. The axial plane was landmarked in all subjects at the center of the forehead, 1 cm above the eyebrows to standardize head position from scan to scan. The forehead was affixed with adhesive tape to the MR bed, and neck support was provided as needed. A neuroradiologist reviewed baseline and posttreatment structural MRI data for potential exclusionary head and brain pathology.

A FAST 3D SPGR sequence was used to acquire volumetric data for cerebrospinal fluid (CSF) correction (axial acquisition; repetition time [TR]=12.6 ms, echo time [TE]=5.6 ms, flip angle=15°, voxel dimensions=0.49×0.49×1.5 mm). Voxel positioning followed a systematic approach during all scans (Figure 1). For the midline anterior cingulate cortex (ACC) voxel, a reference slice was taken from an axial cut approximately 1 cm above the genu of the corpus callosum, demonstrating a continuous view of the anterior and posterior horns of the lateral ventricles. On this reference image, an 8-cm³ voxel (2×2×2 cm) of predominantly gray (prefrontal) matter was centered on the frontal interhemispheric fissure. The posterior margin of the voxel was placed immediately anterior to the genu of the corpus callosum in an area corresponding to the pregenual ACC (Brodmann area 24a, 24b, and 32), as described by Vogt and Vogt (Vogt and Vogt, 2003). For the left dorsolateral prefrontal cortex voxel (L-DLPFC), a reference coronal oblique localizer slice was positioned on the sagittal anatomical images such that it was positioned perpendicular to the average plane of the corpus callosum, and the posterior margin of the slice was located immediately anterior to the anterior-most portion of the genu of the corpus callosum. On this reference image, an 8-cm³ voxel (2×2×2 cm) encompassing the L-DLPFC was placed such that: 1) the superolateral corner of the voxel abutted, but did not include, the skull, 2) the medial margin of the voxel excluded the medial frontal cortex, and 3) the voxel was placed as superiorly as

possible given constraints 1 and 2. This positioning typically includes the superior frontal sulcus and large portions of the superior and middle frontal gyri, containing Brodmann's areas 9 and 46.

While many different spectroscopy sequences are available for measuring Glu and Gln, most commonly short-echo-time (TE=30–35ms) PRESS, we wanted to use sequences that were 1) optimized for measuring each metabolite, and 2) had excellent test-retest variability as this was a longitudinal trial. Based on the prior literature (Hancu, 2009; Hancu and Port, 2011), we chose two different ¹H-MRS sequences for our Glu and Gln measurements, each with its own strengths. A TE-optimized PRESS sequence was used to measure both Glu and Gln (PROBE-P PRESS; TE=80 ms, TR=2000 ms, No. of excitations=8, No. of acquisitions=128) (Hancu and Port, 2011). A 2-dimensional J-resolved averaged PRESS sequence was used with the goal of collecting an optimized measure of Glu (2DJ PRESS; TE=35–195 ms in 16 steps, TR=2000 ms, excitations=8) (Hurd et al., 2004; Adalsteinsson et al., 2004).

2.4. Reconstruction and quantification of spectra

Spectroscopic imaging data were transferred to a Sun workstation running SAGE-IDL (GE Medical Systems). Data integrity was verified visually; scans with artifact were excluded from the study. A quantitative analysis of brain metabolites was performed using the LC Model software (Figure 2). Basis sets for both the 3T-PRESS and 3T-2DJ were provided by the vendor. The lower bound of measurement error for Glu quantification was a Cramer-Rao lower bound of 20 or less. For Gln quantification, the lower bound measurement error was relaxed to 30 or less to optimize both limited data and goodness of fit (Bustillo et al., 2014; Kreis, 2015).

2.5. CSF correction technique

The SPGR anatomical data were segmented into gray matter, white matter, and CSF using a technique modified from a previous study (Port et al., 2008) revised to use the FSL package from FMRIB Oxford (Smith et al., 2004). Briefly, SPGR data were converted into NIFTI format using `mri_convert`. The T1 volume was skull-stripped using BET, then segmented into gray matter, white matter, and CSF using FAST with default parameters. The segmented data were then overlaid with the voxel location using in-house software, and the number of pixels of each tissue type within the voxel were counted. These counts were then normalized to the total number of pixels within the voxel to arrive at the fraction of each tissue within the ¹H-MRS voxel. The tissue volume corrected metabolite concentrations, $[M]_{TVC}$, were then calculated by taking the measured metabolite concentration, $[M]_M$, and applying a correction factor as follows:

$$[M]_{TVC} = [M]_M \times (1/[1 - F_{CSF}])$$

where F_{CSF} is fraction of CSF. This generated “absolute” (vs relative to creatine) metabolite concentrations in “institutional units” specific to our scanner and technique. These CSF-corrected metabolite concentrations were used for all statistical analyses. This approach is in

keeping with our prior methodology and mitigates the potential limitations of the common method of referencing creatine levels (Frye et al., 2007; Port et al. 2008).

Adolescent participants underwent this entire MRI/¹H-MRS procedure at baseline, after completion of up to 30 rTMS treatment sessions over 6 to 8 weeks or exit from the study, and at a 6-month follow-up visit. Two discrete Gln/Glu ratio estimations were determined for each brain region. This included absolute concentrations of Gln and Glu collected from the TE-optimized PRESS sequence (termed *Gln/Glu ratio PRESS*) and a second ratio that consisted of an absolute Gln concentration collected with the TE-optimized PRESS sequence over an absolute Glu concentration collected with the 2DJ PRESS sequence (termed *Gln/Glu ratio 2DJ*).

2.6. Statistical analysis

Continuous variables of demographics and baseline clinical characteristics of the sample were described with means (SDs). Categorical variables were described with frequencies and percentages.

A completely within-subjects linear mixed model analysis of repeated measures was used to examine the change in each Gln/Glu ratio in the ACC and L-DLPFC over 6 to 8 weeks of acute rTMS treatment and throughout the 6-month follow-up period. A separate mixed model analysis was conducted on each of the Gln/Glu ratio measurements. Restricted maximum likelihood estimation, type 3 tests of fixed effects, and generalized least squares (LS) were used, with the Kenward-Roger correction (Kenward and Roger, 1997) applied to the spatial power covariance structure. A weight statement was also included in the mixed model analysis to account for the varied number of rTMS sessions completed by each of the participants (the weight was calculated as the number of rTMS sessions completed divided by the total number of possible sessions [$n=30$]). All youth were included in the weighted analysis, but weighted in proportion to the number of rTMS sessions they attended (e.g., 30/30, 29/30, 17/30, 5/30, and 1/30). Thus, as a measure of precision, the weighted analysis gives more weight to estimates that included a greater number of rTMS sessions attended and less weight to estimates that included a fewer number of rTMS sessions attended. Changes in each of the Gln/Glu ratio measurements over the acute rTMS treatment (baseline to posttreatment) and 6-month follow-up periods (posttreatment to follow-up and baseline to follow-up) were examined using LS mean contrasts from the mixed model, and *P* values associated with the tests of the mean contrasts were adjusted for multiple comparisons using the Tukey-Kramer method in SAS (SAS Institute, Inc). Hedges' *g* (Hedges and Olkin, 1985) was also calculated to estimate effect sizes for the above-mentioned within-subjects LS mean contrasts.

A completely within-subjects linear mixed model repeated measures analysis was also used to examine the relationship between each Gln/Glu ratio measurement and CDRS-R total score (depressive symptoms) over the acute rTMS treatment and follow-up periods. A separate mixed model analysis was conducted on each of the Gln/Glu ratio measures, with CDRS-R total score included as a time-varying covariate. Restricted maximum likelihood estimation was used, with the Kenward-Roger correction (Kenward and Roger, 1997) applied to the spatial power covariance structure. A weight statement was also included to

account for the varied number of rTMS sessions completed by the participants. The parameter estimate (regression coefficient) was interpreted from the solution for fixed effects in the mixed model analysis.

In a sensitivity analysis, a linear mixed model similar to that described above was used to examine the main effect of response status as well as the response $\times$ time interaction on the change in each Gln/Glu ratio in the ACC and L-DLPFC over 6 to 8 weeks of acute rTMS treatment and throughout the 6-month follow-up period. Response was operationally defined as CGI-I of 1 or 2 (very much or much improved) and CDRS-R total < 40 at the end of the 6- to 8-week acute rTMS treatment period (or upon exit from the study). Youth with a CGI-I > 2 and CDRS-R total ≥ 40 at the end of the 6- to 8-week acute rTMS treatment (or upon exit from the study) were considered non-responders.

Finally, a 2-way random effects intraclass correlation coefficient (ICC) was used to evaluate the stability or consistency of the brain measurements across the 3 scans: within each brain region (ACC and L-DLPFC) for each measurement technique (PRESS and 2DJ). Specifically, this was accomplished by evaluating, via the ICC, whether the percent CSF averages were stable across the 3 scans (within each brain area for each measurement technique).

We performed the statistical analyses using SAS software, version 9.4 (SAS Institute, Inc). The procedures of PROC MIXED in SAS software were used to conduct the mixed model analysis. The α level was set at .05 (2-tailed); to address multiple testing, where applicable, P values were adjusted using the false discovery rate (FDR) procedure (Benjamin and Hochberg, 2012).

3. Results

3.1. Participant characteristics

The demographics of the study cohort are shown in Table 1. The mean number of rTMS sessions completed was 23.2 (11.4) (range, 1–30); 6 patients completed all 30 sessions, and 1 each completed 29, 17, 5, and 1 sessions. Of note, CDRS-R scores in the cohort declined considerably following rTMS therapy, and this decline decreased even further by the 6-month followup time point compatible with a durable response. As specified by the protocol participants were on a stable dose of an antidepressant throughout the rTMS trial.

3.2. Tissue segmentation

The reproducibility of the voxel placement over time, as assessed by brain CSF measurements within an individual across the 3 scans, was excellent. Specifically, the ICC for F_{CSF} in the L-DLPFC voxel was 0.80, while for the ACC voxel the ICC for F_{CSF} was 0.94.

3.3. Change in glutamatergic neurotransmission

The mean and standard deviation of the raw metabolite levels for each time point and region are presented in Table 2. Pairwise student's t-test comparisons by region and time point did not reveal any statistically significant differences. Note that while Glu levels measured with

TE-optimized PRESS gradually change over time in each region, Glu levels measured with 2DJ PRESS have more variability. We hypothesize that this finding results from a combination of measurement variability as well as the small number of subjects within the study.

The linear mixed model analyses of repeated measures showed that Gln/Glu ratios increased in the ACC and L-DLPFC during acute rTMS treatment as well as through the 6-month follow-up period (Figure 3). Over the 6-month follow-up, the increase in the Gln/Glu ratio PRESS in the ACC was significant ($F=5.32$; $df=2,10$; raw $P=.02$; FDR $P=.04$), but the increases in the Gln/Glu ratios PRESS and 2DJ in the L-DLPFC did not reach significance (both FDR $P=.08$) (Table 3). There was no significant increase in the Gln/Glu ratio 2DJ in the ACC. To evaluate the timing and pattern of increase in the Gln/Glu ratios in the ACC and L-DLPFC, we examined the LS mean contrasts and respective effect size estimates (Hedges' g) of the Gln/Glu ratio change during the acute rTMS treatment (baseline to posttreatment) and 6-month follow-up periods (posttreatment to follow-up and baseline to follow-up). As shown in Table 4, each of the Gln/Glu ratios in the ACC and L-DLPFC increased during the acute rTMS treatment and continued to increase throughout follow-up. The Gln/Glu ratio PRESS in the ACC had the largest increase throughout the 6-month follow-up, with effect sizes (Hedges' g) of 1.064 (baseline to posttreatment), 1.242 (posttreatment to follow-up), and 1.474 (baseline to follow-up). When we conducted separate linear mixed model analyses, similar to that described above, with age and baseline CDRS-R total score included as covariates, the basic pattern of results did not change (results not reported).

3.4. Relationship between glutamatergic neurotransmission and depressive symptoms

We examined the relationship between each Gln/Glu ratio and CDRS-R total score (depressive symptoms) during the treatment and follow-up periods. The linear mixed model repeated measures analysis revealed a negative (inverse) linear relationship between the CDRS-R total score and the ACC Gln/Glu ratio 2DJ ($b = -0.00014$; 95% CI, $= -0.00045$ to 0.00018 ; raw $P=.37$; FDR $P=.37$), the ACC Gln/Glu ratio PRESS ($b = -0.00123$; 95% CI, $= -0.00277$ to 0.00029 ; raw $P=.10$; FDR $P=.20$), the L-DLPFC Gln/Glu ratio 2DJ ($b = -0.00017$; 95% CI, -0.00043 to -0.00008 ; raw $P=.16$; FDR $P=.21$), and the L-DLPFC Gln/Glu ratio PRESS ($b = 0.00127$; 95% CI, -0.00207 to -0.00048 ; raw $P=.003$; FDR $P=.01$). The direction (inverse relationship) of the regression coefficients suggests that the mean change in each Gln/Glu ratio increased as depression severity decreased. In other words, throughout the 6-month follow-up period, we estimated that a 1 scale unit increase (or decrease) in the CDRS-R total score (depression severity) was related to a mean decrease (or increase) in each Gln/Glu ratio.

3.5 Sensitivity Analysis for Change in Glutamatergic Neurotransmission

We implemented a linear mixed model analysis of repeated measures to examine the main effect of response status as well as the response $\times$ time interaction effect on the change in each Gln/Glu ratio in the ACC and L-DLPFC over 6 to 8 weeks of acute rTMS treatment and throughout the 6-month follow-up period. We found no reliable effects or interpretable patterns between the two response groups on any of the Gln/Glu measures (likely, in part,

because of the small sample size per group—6 responders and 4 non-responders; results not reported).

4. Discussion

In 10 adolescents with treatment-refractory depression, up to 30 sessions of high-frequency rTMS produced increases in the Gln/Glu ratio in the ACC and DLPFC over 6-months' follow-up, as assessed with 2 different ¹H-MRS sequences, but only reached statistical significance in the ACC with a PRESS sequence. Furthermore, throughout the 6-month follow-up, depression symptom severity assessed with the CDRS-R had an inverse relationship with Gln/Glu ratios, which suggests that Gln/Glu ratios increased in conjunction with depressive symptomatic improvement. To our knowledge, this is the first attempt to systematically quantify Gln/Glu changes in conjunction with high-frequency rTMS.

Clinical research studies using ¹H-MRS in depressed patients consistently demonstrate abnormalities in Glu metabolites (Maddock and Buonocore, 2012). Although this is less well studied, extant evidence suggests that cortical Glu metabolites are decreased in children and adolescents with MDD (Rosenberg et al., 2005; Kondo et al., 2011). An enhanced understanding of brain-based therapeutic interventions in developing adolescents is an ethical, clinical, and scientific priority (Krishnan et al., 2015). Studies of neurophysiological measures before and after treatment could optimize the delivery of standard treatments and hone the development of novel brain-based interventions for psychiatric disorders in young persons.

At present, noninvasive brain stimulation therapies such as rTMS show potential as safe and effective treatments for adolescent depression (Donaldson et al., 2014). The antidepressant mechanism of action for high-frequency rTMS remains elusive. A prior ¹H-MRS study of young adults with MDD by Zheng and colleagues (Zheng et al., 2010) suggested that response was associated with an increase in myoinositol in the L-DLPFC. The authors posited that high-frequency rTMS sessions may modulate aberrant glial function in depressed patients (Zheng et al., 2010). Yang and colleagues (Yang et al., 2014) recently examined short-echo ¹H-MRS measures of the L-DLPFC in 6 young patients before and after three weeks of high-frequency rTMS. Those responding to treatment demonstrated an increase in Glu quantification in the L-DLPFC, whereas nonresponders showed a decrease. Our findings in a small sample of depressed adolescents undergoing six weeks of high-frequency rTMS also suggest that cortical glutamate metabolites are modulated with rTMS treatment. In the present study, ¹H-MRS methodology and the directionality of glutamate changes diverged from the work of Yang and colleagues (Yang et al., 2014). Both studies should be viewed as preliminary and interpreted judiciously.

A prior study of adults by Luborzewski and colleagues (Luborzewski et al., 2007) demonstrated that those responding to high-frequency rTMS had decreased Glu levels in the L-DLPFC, which increased with effective treatment. High-frequency rTMS may exert therapeutic effects in depression through modulation of Glu neurotransmission or glial functioning. Baseline measures of metabolites with ¹H-MRS may serve as predictors of treatment response. Given the time and potential financial burden associated with an rTMS

treatment course, predictive biomarkers are critical to identify patients most likely to benefit (Salomons et al., 2014; Liston et al., 2014). However, current data are limited, and larger trials to develop and validate predictive Glu biomarkers of treatment response in adolescents are imperative (Donaldson et al., 2014; Yang et al., 2014).

It is not surprising that all of our basic t-tests and most of the mixed effect model analyses failed to reach statistical significance despite clear visual trends. Glutamine remains extremely challenging to measure, in part due to its low concentration (2–4 mM) and in part due to its large spectral overlap with other brain metabolites (Hancu and Port 2011). Others have developed different acquisition and analysis techniques to try to improve quantification, including optimizing PRESS parameters (Hancu and Port, 2011), averaging the spectra from a 2D J-resolved PRESS acquisition (2D J-averaged PRESS) (Hurd et al. 2004), simultaneously fitting each spectrum from a 2D J-resolved PRESS acquisition (ProFit) (Schulte and Boesiger, 2006), or separately fitting each spectrum from a 2D J-resolved PRESS acquisition and summing the results (Jensen et al, 2009). While each method has its strengths and challenges, none is clearly superior to the other. As such, there remain significant methodological improvements to be made regarding glutamine quantification.

We chose two different MRS sequences for measuring glutamate with the hope of determining if one method is superior to the other. PRESS with TE=80, while shown to be optimal for Gln measurement (Hancu and Port 2011), was also reasonably reliable for Glu measurement (Hancu 2009). In contrast, the 2D J-averaged PRESS technique (Hurd et al 2004) is optimized for measuring Glu as it effectively cancels out the coupled resonances of Gln and GABA, resulting in a “pseudo-singlet” at 2.35 ppm that is easily quantified due to the smoother baseline. We found that both Glu measurements yielded similar visual results (Figure 3), though the TE=80 PRESS sequence did yield the statistically significant finding. As a side note, it is possible to extract a Gln measure from the 2D J-averaged PRESS spectrum; a Gln “pseudo-singlet” is present at 3.75 ppm, but this peak is often contaminated by overlap from residual contributions of both myo-inositol and Glu (Jensen et al., 2009). As such, we did not use the Gln measurement from the 2DJ sequence.

This study has a few limitations. Our results must be examined with caution in the context of a nonrandomized study with a small sample size and no control group. Concurrent medications may have affected spectroscopic findings in this small sample. We did not measure blood levels of medications. All participants were taking a stable dose of various antidepressants at baseline and throughout treatment, but these agents were diverse. Sessions of rTMS may have affected other neurochemicals and brain regions. Resolving Gln and Glu at 3T is challenging (Bustillo et al., 2014; Strawn et al., 2012). We used optimized PRESS and 2DJ sequences with acknowledged strengths (Hancu and Port, 2011; Port et al., 2008). For Gln, Cramer-Rao lower bound criteria were relaxed to 30 or less, as in previous studies, to optimize limited data (Bustillo et al., 2014; Kreis, 2015). However, this relaxed Cramer-Rao lower bound criteria for Gln is a significant limitation of our pilot work. Although it is encouraging that Gln/Glu ratios increased slightly over acute treatment and follow-up in 2 brain regions with 2 spectroscopic techniques, not all the findings were statistically significant and the present Gln results must be interpreted with prudence. A relative strength

of this study, however, is longitudinal ^1H -MRS data at 3 time points, including a longer-term follow-up session at 6 months.

In summary, this is the first study, to our knowledge, to demonstrate increases in the cortical Gln/Glu ratio in adolescents with treatment-refractory depression undergoing high-frequency rTMS. The Gln/Glu ratios also had an inverse relationship with symptom severity throughout the 6-month follow-up. Gln/Glu ratios increased as depression severity decreased. Successful rTMS treatment of adolescent depression may modulate glial cells and Glu neurochemistry. Young persons with lower baseline cortical Gln/Glu ratios in the context of treatment-refractory depression may be preferred candidates for high-frequency rTMS. Further work with larger samples and control groups will be necessary to develop a biomarker for validation. These efforts also have the potential to inform the field on Glu neurochemistry, glial cell function, and the pathophysiology of depression during neurodevelopment.

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Abbreviations

ACC	anterior cingulate cortex
CDRS-R	Children's Depression Rating Scale-Revised
CSF	cerebrospinal fluid
FAST	Fourier acquired steady state
FDR	false discovery rate
Gln	glutamine
Glu	glutamate
^1H-MRS	proton magnetic resonance spectroscopy
ICC	intraclass correlation coefficient
L-DLPFC	left dorsolateral prefrontal cortex
LS	least squares
MDD	major depressive disorder
MRI	magnetic resonance imaging
PRESS	Point resolved spectroscopy

SPGR	spoiled gradient recall
rTMS	repetitive transcranial magnetic stimulation
TE	echo time
TR	repetition time

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Highlights

- Aberrant glutamate neurotransmission may play a role in adolescent depression
- rTMS is a promising new treatment
- Ten adolescents undergoing rTMS were assessed with ^1H -MRS
- Gln/Glu ratios increased in the ACC and LDLPFC

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Figure 1.

Locations of the two sampled MRS voxels. A) The anterior cingulate cortex (ACC) voxel is placed in the midline to sample pregenual anterior cingulate cortex (Brodmann's areas 24a, 24b, and 32). B) The left dorsolateral prefrontal cortex voxel (L-DLPFC) voxel is placed to sample dorsolateral prefrontal cortex and subjacent white matter (Brodmann's areas 9 and 46).

Figure 2.

Representative TE-optimized PRESS spectrum obtained from the L-DLPFC. A) Fitted LCModel spectrum (red) overlaid on the source spectrum (black). B) Individual fit of the Glu metabolite. C) Individual fit of the Gln metabolite. Note that the area under the curve is significantly less than for Glu due to the relatively low concentration of Gln. D) Residual spectrum after subtracting the fitted spectrum from the original spectrum.

Figure 3.

Change in the Gln/Glu ratio over time. A) Ratio assessed using the 2D J-averaged PRESS (2DJ) technique for measuring Glu. B) Ratio assessed using the TE-optimized PRESS technique (P80) for measuring Glu. Note the visually similar trend towards increasing Gln/Glu following treatment and at the 6-month follow-up time point regardless of the MRS method used to measure glutamate. The scale is different due to the different quantification units provided by LCModel.

Table 1**Patient Characteristics**

Characteristic	Value (N=10)^a
Age, y, M±SD	15.4 (1.2)
White, N (%)	10 (100)
Sex, N (%)	
Male	6 (60)
Female	4 (40)
Comorbidities	
ADHD	3(30)
First MDD episode, N (%)	3 (30)
Responders, N (%)	6 (60)
Current MDD episode duration, mo, M±SD	24.3 (22.4)
Age at MDD onset, y, M±SD	12.2 (1.7)
Duration of illness, y, M±SD	3.1 (1.6)
CDRS-R total score, M±SD	
Baseline	62.9 (8.2)
Posttreatment	41.8 (13.2)
6-Month follow-up	34.2 (15.3)
CGI score at baseline, M±SD	5.4 (0.51)
No. of rTMS sessions, M±SD	23.2 (11.4)

Abbreviations: ADHD, Attention-deficit/hyperactivity disorder, CDRS-R, CGI, Clinical Global Impression, Children's Depression Rating Scale-Revised; MDD, major depressive disorder; rTMS, repetitive transcranial magnetic stimulation;

^aValues are mean (SD) or No. of patients (%).

Table 2

Mean raw metabolite values by region and time point

Region	Time	Gln/Glu PRESS	Gln/Glu 2DJ	Gln PRESS	Glu PRESS	Glu 2DJ
ACC						
	Baseline	0.201 ± 0.003	0.038 ± 0.004	2.420 ± 0.279	11.265 ± 2.282	64.138 ± 11.810
	12 weeks	0.250 ± 0.052	0.047 ± 0.014	2.944 ± 0.562	12.396 ± 2.340	62.386 ± 10.158
	6 months	0.285 ± 0.051	0.050 ± 0.008	3.728 ± 1.009	13.106 ± 3.637	67.063 ± 6.966
L-DLPFC						
	Baseline	0.224 ± 0.028	0.044 ± 0.009	2.865 ± 0.484	12.797 ± 1.641	66.031 ± 5.802
	12 weeks	0.244 ± 0.037	0.045 ± 0.008	2.816 ± 0.421	11.672 ± 1.288	63.411 ± 9.979
	6 months	0.267 ± 0.035	0.052 ± 0.011	3.175 ± 0.444	11.595 ± 1.630	65.881 ± 8.256

Table 3

Least Squares Means for Glutamatergic Neurotransmission

Voxel Location and Sequence	Least Squares Mean (SE) of Gln/Glu Ratio, n			Overall ^a	
	Baseline	Posttreatment ^b	6-Month Follow-Up	F Statistic ^c	P Value (FDR) ^d
ACC					
2DJ	0.0392 (0.0049), 5	0.0462 (0.0038), 7	0.0482 (0.0046), 5	F(2,10.7) =0.91	.43 (.43)
PRESS	0.1994 (0.0206), 5	0.2451 (0.0145), 8	0.2949 (0.0194), 5	F(2,10.0) =5.32	.02 (.04)
L-DLPFC					
2DJ	0.0434 (0.0028), 10	0.0443 (0.0030), 8	0.0556 (0.0045), 4	F(2,12.0) =3.17	.07 (.08)
PRESS	0.2261 (0.0105), 10	0.2511 (0.0114), 8	0.2748 (0.0175), 4	F(2,14.3) =3.02	.08 (.08)

Abbreviations: ACC, anterior cingulate cortex; 2DJ, absolute Gln concentration collected with the echo time-optimized PRESS sequence over an absolute Glu concentration collected with the 2D J-resolved averaged PRESS sequence; FDR, false discovery rate; L-DLPFC, left dorsolateral prefrontal cortex; PRESS, absolute concentrations of Gln and Glu collected from the echo time-optimized PRESS sequence.

^a Baseline to 6-month follow-up.

^b Post repetitive transcranial magnetic stimulation treatment was at 6 weeks for 4 patients and 8 weeks for 6 patients.

^c The F statistic was used to test for the omnibus (overall) mean change in each of the glutamatergic neurotransmission outcomes over time.

^d FDR-adjusted P value for the test of the within-subjects time effect.

Table 4
Difference of Least Squares Means and Effect Sizes for Gln/Glu Ratio Over Time

Voxel Location and Sequence	Baseline to Posttreatment ^a			Posttreatment ^a to 6-Mo Follow-up			Baseline to 6-Mo Follow-up		
	<i>P</i> value ^b	<i>t</i> (SE)	Effect Size ^c	<i>P</i> value ^b	<i>t</i> (SE)	Effect Size ^c	<i>P</i> value ^b	<i>t</i> (SE)	Effect Size ^c
ACC									
2DJ	.28 (.52)	0.0069 (.0062)	0.573	.74 (.94)	0.0019 (.0058)	0.188	.21 (.42)	0.0089 (.0069)	0.593
PRESS	.08 (.18)	0.0457 (.0239)	1.064	.05 (.12)	0.0497 (.0228)	1.242	.005 (.01)	0.0955 (.0291)	1.474
L-DLPFC									
2DJ	.78 (.96)	0.0009 (.0032)	0.153	.03 (.07)	0.0112 (.0046)	1.176	.03 (.07)	0.0121 (.0051)	1.036
PRESS	.13 (.28)	0.0250 (.0155)	0.824	.27 (.51)	0.0236 (.0209)	0.509	.03 (.07)	0.0486 (.0208)	0.963

Abbreviations: ACC, anterior cingulate cortex; 1, least squares mean for posttreatment minus baseline; 2, least squares mean for 6-month follow-up minus posttreatment; 3, least squares mean for 6-month follow-up minus baseline; 2DJ, absolute Gln concentration collected with the echo time-optimized PRESS sequence over an absolute Glu concentration collected with the 2D J-resolved averaged PRESS sequence; L-DLPFC, left dorsolateral prefrontal cortex; PRESS, absolute concentrations of Gln and Glu collected from the echo time-optimized PRESS sequence.

^aPost repetitive transcranial magnetic stimulation treatment was at 6 weeks for 4 patients and 8 weeks for 6 patients.

^b*P* value using *t* test, in a within-subjects linear mixed model analysis of repeated measures, was used to test for differences of least squares means between periods (*P* values in parentheses were adjusted for multiple testing using the Tukey-Kramer procedure).

^cHedges' *g*.