

Altered Glutamate and Regional Cerebral Blood Flow Levels in Schizophrenia: A ¹H-MRS and pCASL study

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The neurobiology of schizophrenia (SZ) may be altered in older versus younger adults with SZ, as less frequent episodes of symptom exacerbation and increased sensitivity to medications are observed in older age. The goal of this study was to examine the effect of age and diagnosis on glutamate and cerebral blood flow (rCBF) in adults with SZ and healthy controls. Young and older adults with SZ and healthy controls were recruited to participate in this study. Participants completed a neuropsychological battery and neuroimaging that included optimized magnetic resonance spectroscopy to measure anterior cingulate (AC) glutamate (Glu) and glutamine (Gln) and arterial spin labeling evaluation for rCBF. Regression analyses revealed significant effects of age with Glu, Gln, Gln/Glu, and AC white matter (WM) rCBF. Glu and WM rCBF decreased linearly with age while Gln and Gln/Glu increased linearly with age. Glu was lower in adults with SZ compared with healthy controls and in older adults versus younger adults but there was no interaction. Glu and WM rCBF were correlated with the UCSD Performance-Based Skills Assessment (UPSA) and processing speed, and the correlations were stronger in the SZ group. In the largest sample to date, lower Glu and elevated Gln/Glu levels were observed in adults with SZ and in older subjects. Contrary to expectation, these results do not show evidence of accelerated Glu aging in the anterior cingulate region in SZ compared with healthy controls.

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INTRODUCTION

The neurobiology of schizophrenia (SZ) may be altered in older versus younger adults with SZ since older adults with SZ have less frequent episodes of symptom exacerbation and increased sensitivity to medications (Jeste and Maglione, 2013). Post-mortem studies of several brain regions including the anterior cingulate have shown altered regional morphologies due to aging (Torii *et al*, 2012) and glutamatergic receptor expression (Zavitsanou *et al*, 2002). However, there have been very few studies that examine the effects of aging in schizophrenia using advanced magnetic resonance techniques at 3 T. Glutamate (Glu), the primary excitatory neurotransmitter in the human brain, has been implicated in the pathophysiology of SZ since Glu N-methyl-D-aspartate receptor (NMDAR) antagonists such as ketamine and phencyclidine (PCP) induce positive, negative, and

cognitive symptoms similar to SZ (Krystal *et al*, 1994). Glu can be non-invasively measured *in vivo* using ¹H-MRS, and this technique has been utilized in numerous studies to investigate Glu in the medial frontal lobe, including the anterior cingulate (AC), in various stages of the illness: prodrome, first episode, and chronic (Bustillo *et al*, 2010; Egerton *et al*, 2012; Natsubori *et al*, 2014; Rowland *et al*, 2013; Tayoshi *et al*, 2009; Theberge *et al*, 2002, 2003). A recent meta-analysis showed a decrease in Glu that was potentiated with age as measured with ¹H-MRS in the medial frontal lobe of adults with SZ (Marsman *et al*, 2013).

In conjunction with ¹H-MRS, utilization of arterial spin labeling (ASL) to quantify regional cerebral blood flow (rCBF) may provide additional insight into the pathophysiology of the illness. Blood flow is tightly coupled to metabolism and neural activity in the steady state brain (Busija *et al*, 2007), so that lower rCBF is taken as a marker of diminished neural activity. Previous ASL studies of schizophrenia have shown a relationship between rCBF and positive and negative symptoms as well as processing speed (Horn *et al*, 2009; Pinkham *et al*, 2011; Wright *et al*, 2015), and highlighted a reduction in white matter (WM) perfusion with aging (Wright *et al*, 2014). Thus, these two

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techniques are complementary in that ^1H -MRS provides information about glutamatergic tissue levels and ASL captures rCBF that is modulated by local glutamatergic neurotransmission.

The goals of this study were to examine the effect of age and diagnosis in the largest sample to date on AC Glu and AC rCBF and to examine the relationship between AC Glu and AC rCBF. Glutamine (Gln) is the major metabolite of Glu, and it can also be detected reliably using a specifically optimized MRS technique for detection of these metabolites (Bustillo *et al*, 2015; Wijtenburg *et al*, 2013). In addition, Gln/Glu ratio is thought by some to reflect glutamatergic neurotransmission via glutamate–glutamine (Bustillo *et al*, 2010; Shirayama *et al*, 2010).

Because the spectroscopic voxel contained both gray matter (GM) and white matter (WM), we examined the relationship between Glu and both AC GM rCBF and AC WM rCBF. Single-voxel MRS cannot differentiate between gray and white matter glutamatergic metabolites. Therefore, both GM and WM rCBF were examined. Historically, GM has been linked to Glu neurotransmission; however, emerging evidence highlights the importance of Glu neurotransmission in WM (Kukley *et al*, 2007) and that significant white matter alterations have been observed in SZ (Dias, 2012; Kochunov *et al*, 2014). Overall, we hypothesized that Glu and rCBF would be reduced as a function of age and diagnosis such that older adults with SZ (OSZ) would have the lowest Glu and rCBF levels compared with younger adults with SZ (YSZ), younger healthy adults (YHC), and older healthy adults (OHC).

MATERIALS AND METHODS

Participant Characteristics

This study was approved by the University of Maryland Baltimore Institutional Review Board, and all participants provided informed consent prior to study enrollment. Adults with SZ were evaluated for capacity to consent to ensure that each participant understood the study and its procedures. A total of 188 participants were enrolled in this study: 48 YSZ, 47 OSZ, 54 OHC, and 39 OHC. Age group assignment was determined by median split with the median being 36 years old such that younger adults were of ages 18–36 and older adults were of ages 37–62. There were 167 participants with MRS data and 105 participants with ASL data. Thirty-six of the 167 MRS datasets overlap with a previous study by Rowland *et al* (Rowland *et al*, 2016), and 30 of the 105 ASL datasets overlap with two previous studies by Wright *et al* (Wright *et al*, 2014, 2015). Participant demographics are detailed in Table 1, and a breakdown of the antipsychotic medications being taking by adults with SZ are summarized in Supplementary Table 1. Each patient with SZ was characterized and evaluated with the Structured Clinical Interview for DSM-IV (SCID), the Brief Psychiatric Rating Scale (BPRS), and Brief Negative Symptom Scale (BNSS). Inclusion criteria for adults with schizophrenia and healthy controls were (1) no current or past neurological condition, head trauma, or focal findings on MR scan, (2) no substance abuse in past 6 months or lifetime dependence excluding nicotine, and (3) no contraindication for MRI scanning. Healthy controls were

Table 1 Participant Demographics, Mean \pm Standard Deviation

	YSZ	YHC	OSZ	OHC
Male/Female	34/14	25/29	26/21	25/14
Age (years)	25.2 \pm 4.5	25.2 \pm 4.8	49.5 \pm 5.4	51.2 \pm 5.7
^a Education (years)	13.1 \pm 2.0	14.4 \pm 2.0	12.7 \pm 2.0	15.1 \pm 2.2
Duration of Illness (years)	6.1 \pm 5.8		25.4 \pm 9.3	
^b Smoker (yes/no)	19/29	14/40	21/26	10/29
Psychiatric ratings				
BPRS (total)	33.4 \pm 13.0		33.6 \pm 15.8	
BPRS (positive)	8.5 \pm 4.3		9.0 \pm 5.3	
BPRS (negative)	10.5 \pm 10.4		10.1 \pm 12.0	
BNSS	16.5 \pm 11.1		16.4 \pm 14.5	
Antipsychotic medication				
Typical	2		8	
Atypical	41		32	
Both (typical+atypical)	2		6	
None	4		2	
Other Medications				
Mood Stabilizers	1		6	
Benzodiazepine	8		12	
Anticholinergic	4		7	
Cognitive Measures				
^c Processing speed	58.0 \pm 19.8	73.3 \pm 20.0	47.4 \pm 18.6	61.9 \pm 15.3
^d Verbal working memory	16.4 \pm 4.9	18.6 \pm 4.5	14.6 \pm 5.0	18.1 \pm 4.5
^e UPSA	90.0 \pm 19.4	103.8 \pm 6.5	85.0 \pm 14.6	102.9 \pm 9.6

Abbreviations: OHC, older healthy controls; OSZ, older adults with SZ; YHC, young healthy controls; YSZ, younger adults with SZ.

^aEducation: main effect of diagnosis ($p < 0.05$).

^bSmoker: main effect of diagnosis ($p < 0.05$).

^cProcessing Speed: main effect of age ($p < 0.05$) and diagnosis ($p < 0.05$).

^dVerbal Working Memory: main effect of diagnosis ($p < 0.05$).

^eUPSA: main effect of diagnosis ($p < 0.05$).

included in this study if they did not have a past or current Axis I DSM-IV diagnosis and had no family history of a psychotic disorder. Participants were administered the UCSD Performance–Based Skills Assessment (UPSA) (Patterson *et al*, 2001) to assess functional capacity. Since processing speed and working memory deficits are among the most robust cognitive impairments in schizophrenia (Knowles *et al*, 2010; Lee and Park, 2005), participants were assessed with the Digit–Symbol Coding subtest from the Wechsler Adult Intelligence Scale (WAIS) for processing speed and the Digit–Span Sequencing task from WAIS for verbal working memory (Wechsler, 1997).

Imaging Parameters

Participants were scanned using a Siemens TIM Trio 3 T MR system with a 32-channel phased array head coil located at the University of Maryland Center for Brain Imaging Research. MP-RAGE images were prescribed parallel with the AC–PC line. The spectroscopic voxel was placed in the

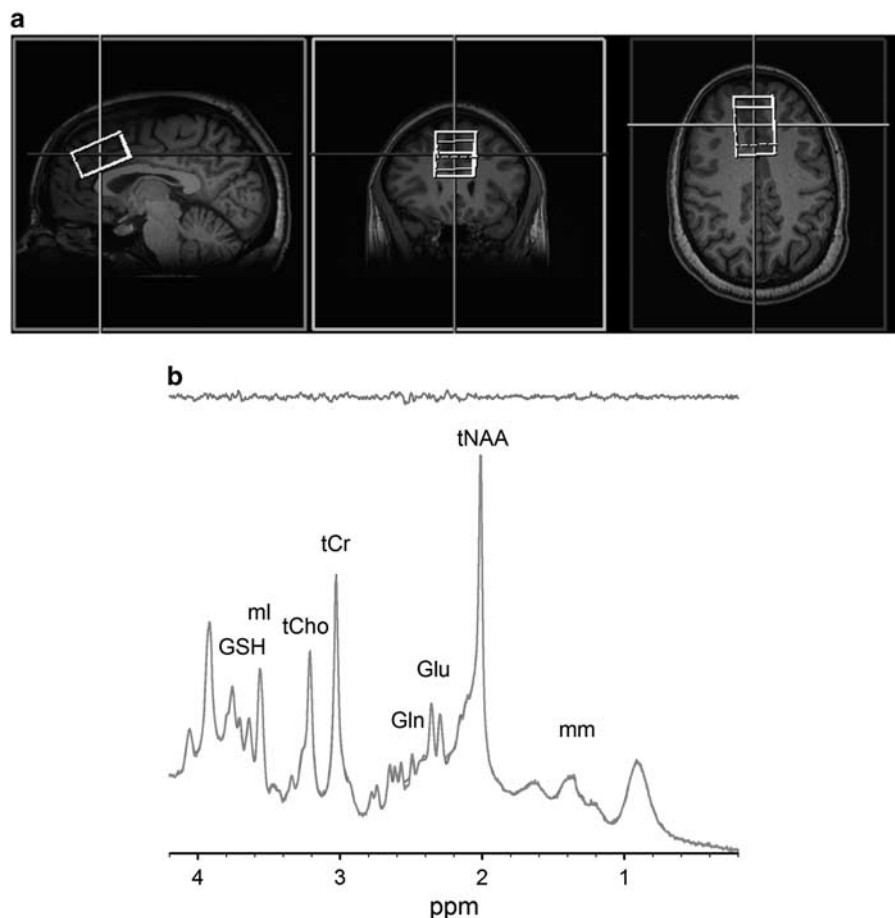


Figure 1 (a) T1-weighted images showing voxel placement in the medial prefrontal lobe. (b) Representative spectra (gray, top) with corresponding LCMoDel fit (red) and residue (gray, bottom) shown above. Examination of the fit residues shows noise with no definitive peaks, showing excellent fits to the data. A full color version of this figure is available at the *Neuropsychopharmacology* journal online.

medial prefrontal region along the midline (Figure 1a). Spectroscopy data were acquired using phase rotation STEAM (PR-STEAM) (Bustillo *et al*, 2015; Wijtenburg *et al*, 2013; Wijtenburg and Knight-Scott, 2011): TR/TM/TE = 2000/10/6.5-ms, VOI ~ 24-cm³ (R-L: 30 mm x A-P: 40mm x F-H: 20mm and fixed across subjects), NEX = 128, 2.5-kHz spectral width, 2048 complex points, and phases: $\varphi_1 = 135^\circ$, $\varphi_2 = 22.5^\circ$, $\varphi_{13} = 112.5^\circ$, $\varphi_{ADC} = 0^\circ$ optimized for detection of Glu (Wijtenburg and Knight-Scott, 2011) and shown to have good reproducibility for Glu and glutamine, separately (Bustillo *et al*, 2015; Wijtenburg *et al*, 2013). A water reference (NEX = 16) was also acquired for phase and eddy current correction as well as quantification. Spectroscopy post-processing methods can be found in the supplement. Metabolite levels were reported in institutional units, and all metabolites except Gln with percent standard deviation Cramer Rao Lower Bounds (CRLBs) $\leq 20\%$ were included in statistical analyses. For Gln, metabolites with CRLB $\leq 25\%$ were included in statistical analyses, which are consistent with published reports (Bustillo *et al*, 2014; Shirayama *et al*, 2010). For measurement of rCBF, a pseudo-continuous arterial spin labeling (pCASL) sequence was applied with the following parameters: TR/TE = 4000/16 ms, FOV = 220 × 220 mm, Number of slices = 23, Slice thickness = 5 mm, Voxel size = 3.4 × 3.4 × 5.0 mm³, Bandwidth = 1594 Hz/pixel,

136 measurements, labeling offset = 90 mm, labeling duration of 1.85 s, and post labeling delay of 0.93 s (Wu *et al*, 2007). Arterial spin labeling post-processing methods can be found in the supplement.

Statistical Analyses

Demographic, clinical, and cognitive variables were analyzed in SPSS version 23.0 using chi-square for categorical variables and regression models for continuous variables with significance set at $p < 0.05$. To examine the effects of age and diagnosis, a linear regression model incorporating age, diagnosis, and the interaction term was applied to main outcome variables: glutamatergic metabolites (Glu, Gln, and Gln/Glu) and CBF measures (AC GM rCBF, cingulated WM rCBF, and whole brain (WB) CBF). If the regression model was not significant, then each term was evaluated for its significance and the regression model was adjusted accordingly. Age was centered by subtracting out the median age of 36 and then divided by 10 to compute a change in dependent variable per decade in age. Centering the data to the median age shifted interpretation of the regression coefficient for diagnosis to the estimated difference between HC and SZ at the overall median age rather than the difference at age 0. Age divided by 10 shifts the interpretation

Table 2 Mean Metabolite and rCBF for Younger and Older Adults with Schizophrenia and Age-Matched Controls

	Younger schizophrenia		Younger controls		Older schizophrenia		Older controls	
	Mean \pm Std. Dev. (I.U.)	Mean CRLB (%)	Mean \pm Std. Dev. (I.U.)	Mean CRLB (%)	Mean \pm Std. Dev. (I.U.)	Mean CRLB (%)	Mean \pm Std. Dev. (I.U.)	Mean CRLB (%)
tNAA	10.1 \pm 0.7	2.5	10.3 \pm 0.6	2.3	9.33 \pm 0.9	3.1	9.87 \pm 0.7	3.3
Gln	2.34 \pm 0.5	16.3	2.19 \pm 0.4	17.1	2.57 \pm 1.0	17.3	2.39 \pm 0.5	17.4
Glu	9.25 \pm 0.8	4.6	9.54 \pm 0.7	4.4	8.16 \pm 1.0	5.5	8.71 \pm 0.8	5.2
Glx	11.49 \pm 1.1	5.1	11.7 \pm 1.0	4.8	10.6 \pm 1.4	5.8	11.0 \pm 1.1	5.5
tCr	9.12 \pm 0.8	2.6	9.19 \pm 0.6	2.4	9.02 \pm 0.9	2.8	9.49 \pm 0.8	3.2
tCho	1.83 \pm 0.3	4.9	1.82 \pm 0.3	4.5	1.93 \pm 0.3	5	1.89 \pm 0.3	4.7
ml	6.60 \pm 0.7	3.9	6.72 \pm 0.5	3.4	6.82 \pm 0.9	4	7.00 \pm 0.8	3.8
GSH	2.17 \pm 0.2	7.7	2.16 \pm 0.2	7.4	2.07 \pm 0.3	8.6	2.18 \pm 0.3	8.1
Gln/Glu	0.25 \pm 0.06		0.23 \pm 0.04		0.30 \pm 0.09		0.27 \pm 0.06	
AC GM rCBF (ml/100g/min)	55.7 \pm 13.9		58.8 \pm 10.5		54.1 \pm 14.2		54.2 \pm 9.8	
AC WM rCBF (ml/100g/min)	55.7 \pm 13.9		58.8 \pm 10.5		54.1 \pm 14.2		54.2 \pm 9.8	
Whole Brain CBF (ml/100g/min)	15.8 \pm 5.6		16.9 \pm 4.9		12.5 \pm 4.9		15.0 \pm 3.6	

of our estimates from ‘change per year’ to ‘change per 10 years,’ which seems helpful in a cohort whose ages range over 4 decades. Similar exploratory models for the other MRS metabolites were applied as well. Unstandardized coefficients (β) \pm SE, t , degrees of freedom (df), and p -values were reported for regression models. Bonferroni correction for multiple comparisons was set to $p < 0.0083$ ($0.05/6$) for the linear regression analyses. Exploratory regression analyses were conducted for the other six metabolites found in the spectrum with $p < 0.05/6$, and results are summarized in the Supplement.

Pearson product moment correlations were conducted between main outcome variables: glutamatergic metabolites and CBF values. Next, correlation analyses were conducted between glutamatergic metabolites and CBF values with cognitive measures across combined groups and clinical symptoms in the adults with SZ group. If the overall correlations were significant, further correlation analyses were performed on each diagnostic group. Fisher’s Z transformation was used to test the significance of the difference in magnitude of these correlations. Benjamini–Hochberg false discovery rate at $p < 0.05$ was used for correction of multiple correlation analyses between glutamatergic metabolites, CBF metrics, cognitive measures, and clinical symptom assessments. This correction was applied across Table 2 and Supplementary Tables 3 and 4 that shows the uncorrected p -values with significance set at $p < 0.008$ after FDR correction.

RESULTS

Participant Characteristics

Participant demographics, clinical measures, and cognitive measures are summarized in Table 1. Regression results are summarized in the Supplement. Using covariates of smoking status and CPZ had no effect on the study results (Supplementary Information).

Glutamatergic Metabolites

A representative spectrum and corresponding LCModel fit is shown in Figure 1b, highlighting the excellent spectral quality acquired in this study. Mean line width (LW) and signal-to-noise ratio (SNR) as reported by LCModel and mean GM, WM, and CSF proportions are summarized in the Supplementary Table 2. Mean metabolite levels are summarized in Table 2. There were significant effects for GM, WM, and CSF between the groups similar to another study (Bustillo *et al*, 2014); however, these differences in voxel composition are accounted for with partial volume correction applied to the metabolite concentrations as discussed in the Method section. MRS data were not included in further analyses with LCModel reported SNR of < 10 or LW > 0.1 . One of the 167 MRS data sets was excluded due to poor SNR and broad LW. There were no Glu data sets excluded based on $CRLB \leq 20\%$, and there were 16 out of 166 Gln datasets excluded due to $CRLB > 25\%$.

For Glu, examination of regression analyses incorporating age, diagnosis, and the interaction term revealed a significant linear relationship with significant age ($\beta_{age} = -0.39 \pm 0.047$, $t = -8.23$, $df = 165$, $p < 0.001$) and diagnosis effects ($\beta_{dx} = -0.44 \pm 0.13$, $t = -3.47$, $df = 165$, $p = 0.001$) (Figure 2a). Thus, Glu levels, reported in institutional units, change -0.39 per 10 year increase in age and the average distance between HC and SZ Glu levels is -0.44 , suggesting that SZ have lower Glu levels. With $CRLB \leq 25\%$, regression models for age, diagnosis, and interaction revealed a significant linear relationship between age and Gln ($\beta_{age} = 0.082 \pm 0.03$, $t = 2.71$, $df = 149$, $p = 0.007$) (Figure 2b). Thus, Gln levels increase 0.082 per 10 year increase in age, and the average distance between HC and SZ Gln levels is negligible. With $CRLB \leq 20\%$ for Gln, the significant finding of a linear relationship between age and Gln held (Supplementary Information).

For Gln/Glu and age, a linear regression model revealed a significant relationship with age ($\beta_{age} = 0.021 \pm 0.004$,

Table 3 Correlation Summary between Glutamatergic Metabolites and Cerebral Blood Flow

Metabolite	rCBF	Cohort	r	Uncorrected p-value	Adjusted p-value	df
Glu	AC GM	All	0.14	0.15	0.31	103
Glu	AC WM	All	0.42	<0.001 ^a	<0.001	103
Glu	AC WM	HC	0.24	0.089	0.22	50
Glu	AC WM	SZ	0.49	<0.001 ^a	<0.001	51
Glu	AC WM	YSZ	0.52	0.006 ^a	0.033	24
Glu	AC WM	OSZ	0.38	0.052	0.15	25
Glu	WB	All	0.095	0.34	0.51	103
Gln	AC GM	All	-0.032	0.76	0.84	92
Gln	AC WM	All	-0.17	0.11	0.25	92
Gln	WB	All	-0.15	0.16	0.36	92
Gln/Glu	AC GM	All	-0.073	0.48	0.63	92
Gln/Glu	AC WM	All	-0.32	0.002 ^a	0.017	92
Gln/Glu	AC WM	HC	-0.38	0.008	0.039	46
Gln/Glu	AC WM	SZ	-0.26	0.079	0.20	44
Gln/Glu	WB	All	-0.16	0.12	0.26	92

Abbreviations: AC, anterior cingulate; GM, gray matter; Gln, glutamine; Gln/Glu, glutamine to glutamate ratio; Glu, glutamate; HC, healthy controls; OSZ, older SZ; SZ, schizophrenia; WB, whole brain; WM, white matter; YHC, younger HC; YSZ, younger SZ.

^a $p < 0.008$ significant after FDR correction (applied across Table 2 and Supplementary Table 3 and 4).

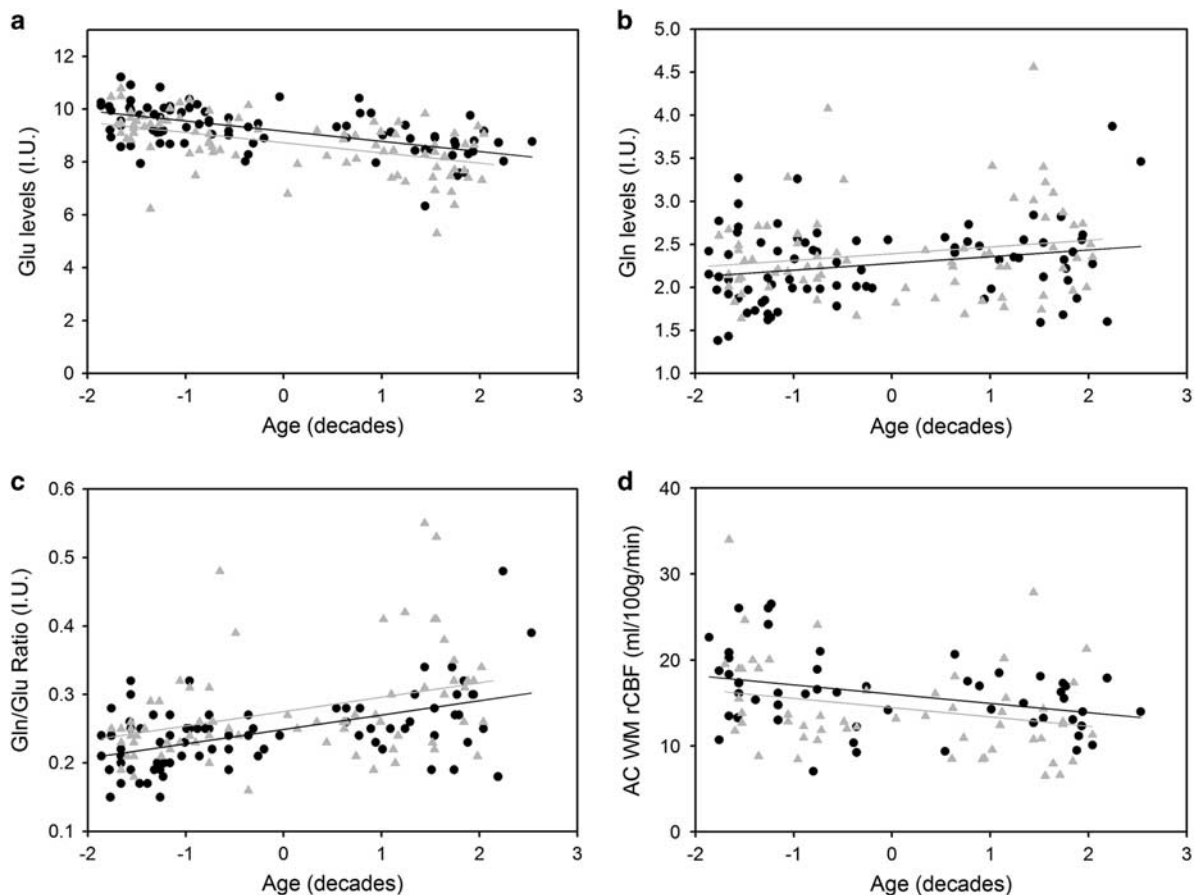


Figure 2 Neuroimaging measures correlated with age by group. Age was centered (median age of 36) and divided by 10 in order to obtain results in reference to changes in measures per decade of age. (a) Plots of Glu levels as a function age in both SZ (▲) and HC (●). The significant linear model with age and diagnosis as main effects revealed lower Glu in SZ than HC and a decrease in Glu levels with aging in both groups. (b) Plots of Gln levels as a function age in both SZ (▲) and HC (●). A linear regression model with age and diagnosis effects revealed that age was the only significant predictor of Gln levels such that Gln increased with age. There was not a significant diagnosis effect as evidenced by similar increasing slope for both groups. (c) Plots of Gln/Glu ratio levels as a function age in both SZ (▲) and HC (●). The significant linear regression model with age and diagnosis as main effects revealed that Gln/Glu is elevated in SZ and increases with aging in both groups. (d) Plots of AC rCBF levels as a function age in both SZ (▲) and HC (●). Application of a linear regression model revealed that rCBF is lower in older adults versus younger adults (as evidenced by the negative slope).

$t=5.79$, $df=149$, $p<0.001$) and diagnosis ($\beta_{dx}=0.026 \pm 0.01$, $t=2.72$, $df=149$, $p=0.007$) effects (Figure 2c). Thus, Gln/Glu levels increase 0.021 per 10 years of life, and the average distance between HC and SZ shows that SZ have higher levels of Gln/Glu than HC. There were no significant interaction effects in any of the three significant linear models for Glu, Gln, and Gln/Glu so the term was removed.

rCBF

Mean rCBF levels are summarized in Table 2. Since the spectroscopic voxel contained both GM (mean across groups ~52%) and WM (mean across groups ~36%), rCBF analyses were conducted for AC GM rCBF and WM rCBF as well as WB CBF, resulting in a total of 105 participants with ASL data. For AC GM rCBF, linear regression analyses with age, diagnosis, and interaction revealed no significant effects ($p=0.20$). Linear regression model analyses between age and WM rCBF revealed a significant linear relationship ($p=0.001$) with age ($\beta_{age}=-1.18 \pm 0.34$, $t=-3.46$, $df=104$, $p=0.001$) and not diagnosis ($p=0.09$; Figure 2d). Thus, WM rCBF decreases every 10 years of age. Linear regression model analyses with age and diagnosis as the main effects for WB CBF were not significant ($p=0.071$). Exploratory analysis of the CBF from the MRS voxel produced similar results (Supplementary Information).

Correlations between Glutamatergic Metabolites and rCBF

Correlation results for glutamatergic metabolites and CBF are summarized in Table 3. Glu was not significantly correlated with AC GM rCBF or WB CBF. Glu and AC WM rCBF were significantly correlated across combined groups ($r=0.42$, $df=103$, $p<0.001$; Supplementary Figure 1). When broken down according to diagnosis, there was a significant correlation in SZ ($r=0.49$, $df=51$, $p<0.001$) and not in HC ($r=0.24$, $df=50$, $p=0.089$), and the difference in magnitude between the two correlations was not significant ($Z\text{-score}=1.45$, $p=0.15$). Further examination into the SZ data revealed a significant relationship between Glu and AC WM rCBF in YSZ ($r=0.52$, $df=24$, $p=0.006$) compared with OSZ ($r=0.38$, $df=25$, $p=0.052$); however, the correlation coefficients were not significantly different ($Z\text{-score}=0.60$, $p=0.55$). Thus, higher Glu was associated with higher AC WM rCBF, and direction of the relationship was similar in HC and SZ. Due to the CRLB cut-off of 25% or less, the number of participants with glutamatergic and CBF data decreased to 94 for Gln and Gln/Glu measurements. There were no significant correlations between Gln and any CBF metrics. For Gln/Glu, there was a significant finding between Gln/Glu and AC WM rCBF ($r=-0.32$, $df=92$, $p=0.002$) across groups (Supplementary Figure 2). The correlation coefficient in HC ($r=-0.38$, $df=46$, $p=0.008$) was significant at trend level compared with the SZ group ($r=-0.26$, $df=44$, $p=0.079$), and the difference in correlation coefficients was not significant ($Z\text{-score}=-0.63$, $p=0.53$). Thus, as Gln/Glu decreases, AC WM rCBF increases and this relationship is similar in both diagnostic and age groups.

Correlations with Cognitive Measures

Correlation results for cognitive measures with glutamatergic metabolites and rCBF levels are summarized in Supplementary Table 3. Across both groups, Glu was moderately significantly correlated with UPSA total score ($r=0.31$, $df=139$, $p<0.001$), processing speed ($r=0.39$, $df=158$, $p<0.001$), and verbal working memory ($r=0.23$, $df=155$, $p=0.005$). Separating by diagnostic groups showed a positive relationship between Glu and UPSA in SZ ($r=0.27$, $df=73$, $p=0.018$) compared with a negative relationship between Glu and UPSA in HC ($r=-0.12$, $df=64$, $p=0.34$), and the Z -score showed that the correlations were significantly different ($Z\text{-score}=-2.31$, $p=0.021$). For processing speed, the strong positive correlation between Glu and processing speed was present in HC ($r=0.28$, $df=78$, $p=0.011$) and SZ ($r=0.36$, $df=78$, $p=0.001$) with the correlations in each group being at trend level (HC) or significant (SZ), but the difference in magnitude of the correlation was not ($Z\text{-score}=-0.51$, $p=0.61$). Further examination into age groups revealed that the relationship between processing speed and Glu was more pronounced in OSZ ($r=0.35$, $df=38$, $p=0.026$) compared with YSZ ($r=0.20$, $df=40$, $p=0.22$). In terms of diagnostic groups, Glu and verbal working memory correlations were not significant in either HC or SZ.

Across both groups, Gln was correlated with UPSA ($r=-0.25$, $df=124$, $p=0.006$) and processing speed ($r=-0.23$, $df=142$, $p=0.005$), but not verbal working memory. When separated by diagnostic group, the correlations between UPSA and Gln were very similar and not significant in HC ($r=-0.23$) and in SZ ($r=-0.21$) with the Z -score revealing no significant difference ($Z\text{-score}=0.12$, $p=0.91$). The relationship between Gln and processing speed was similar in HC ($r=-0.15$) versus SZ ($r=-0.24$) and not significant for either correlation or in terms of the Z -score ($Z\text{-score}=0.53$, $p=0.60$).

For Gln/Glu, there was a significant relationship between Gln/Glu and processing speed ($r=-0.39$, $df=142$, $p<0.001$) and UPSA ($r=-0.37$, $df=124$, $p<0.001$), but not with verbal working memory across groups. In SZ, there was a significant correlation between Gln/Glu and processing speed ($r=-0.37$, $df=67$, $p=0.002$), and a similar relationship was observed in HC ($r=-0.27$, $df=73$, $p=0.018$). When further delineated by age group within the SZ group, OSZ had a trend level negative correlation between processing speed and Gln/Glu ($r=-0.42$, $df=33$, $p=0.012$) while YSZ had a non-significant correlation ($r=-0.22$). Overall, even though the correlation coefficients between processing speed and Gln/Glu in HC versus SZ ($Z\text{-score}=0.66$, $p=0.51$) and YSZ versus OSZ ($Z\text{-score}=0.89$, $p=0.37$) were not significant, there was still a strong negative relationship across groups such that lower processing speed scores were associated with higher Gln/Glu levels. In terms of UPSA, the SZ group had a more negative correlation at trend level ($r=-0.32$, $df=62$, $p=0.01$) compared with HC ($r=-0.15$, $df=60$, $p=0.24$), but the Z -score between correlations was not significant ($Z\text{-score}=-0.99$, $p=0.32$).

In terms of CBF measures, there were no significant correlations between AC GM rCBF with any cognitive measures across all groups. AC WM rCBF was significantly

associated with UPSA ($r=0.29$, $df=93$, $p=0.005$) and at trend level with processing speed ($r=0.26$, $df=101$, $p=0.008$), but not verbal working memory with both diagnostic groups combined. When separated by diagnostic group, UPSA and AC WM rCBF were positively correlated in SZ ($r=0.36$, $df=48$, $p=0.011$) at trend level, and negatively correlated in HC ($r=-0.12$, $df=43$, $p=0.046$), and the two correlations were significantly different between groups ($Z\text{-score}=-2.28$, $p=0.023$). Since processing speed and AC WM rCBF were significant at trend level ($p=0.008$), separation by diagnostic group revealed a significant moderate correlation in SZ ($r=0.37$, $df=50$, $p=0.002$) but not in HC ($r=0.002$, $df=49$, $p=0.99$). The difference in magnitude between the correlations was not significant ($Z\text{-score}=-1.91$, $p=0.056$). When further delineated by age group, while not significant, the association between processing speed and Glu was more pronounced in YSZ ($r=0.42$, $df=24$, $p=0.033$) compared with OSZ ($r=0.22$, $df=24$, $p=0.28$). In terms of WB CBF, there was no association with UPSA, verbal working memory, or processing speed.

Correlations with Clinical Symptoms

Supplementary Table 4 summarizes the correlations between clinical variables, glutamatergic metabolites, and CBF. In SZ, there were no significant correlations between BNSS, BPRS total, BPRS positive, and BPRS negative scores and Glu ($p>0.36$), Gln ($p>0.07$), Gln/Glu ($p>0.16$), AC GM rCBF ($p>0.24$), WM rCBF ($p>0.53$), and WB rCBF ($p>0.17$).

DISCUSSION

This is the largest study to examine glutamatergic and regional cerebral blood flow differences between adults with SZ and healthy controls. This study used a $^1\text{H-MRS}$ technique specifically tailored to detect Glu, and an optimal ASL technique to measure rCBF (Alsop *et al*, 2014; Wijtenburg and Knight-Scott, 2011). The major findings showed adults with SZ had lower Glu than HC and lower Glu and AC WM rCBF levels in older adults versus younger adults irrespective of diagnosis. The inverse was true in terms of Gln/Glu with it increasing with age in both groups and it was higher in SZ compared with HC. Gln levels increased with age regardless of diagnosis. Contrary to expectation (Marsman *et al*, 2013), these results do not show evidence of accelerated Glu or Gln/Glu aging in the anterior cingulate region in SZ compared with healthy controls. These data also suggest that adults with SZ do not experience accelerated aging in AC rCBF.

Few studies have examined the effects of age on Glu in SZ. One study observed that chronic SZ participants had the lowest Glx compared with ultra high risk and first episode participants in the medial prefrontal cortex (Natsubori *et al*, 2014), while another study found reduced Glx in the anterior cingulate and white matter centrum semiovale in SZ but no age effect (Rowland *et al*, 2013). Bustillo *et al* (2014) did not find age or diagnosis effects for anterior cingulate Glu but did find alterations in Gln and Gln/Glu in SZ (Bustillo *et al*, 2014). The reduction in Glu levels in SZ observed in this

study may be potentially explained by a combination of abnormal Glu receptor function and reduced neuropil (Dickstein *et al*, 2007; Rubio *et al*, 2012). Overall, this study provides further evidence of a reduction in Glu with SZ similar to other studies (Natsubori *et al*, 2012, 2003) and with age (Hadel *et al*, 2013).

Glutamine and Gln/Glu were elevated as a function of age and Gln/Glu was higher in schizophrenia. These results are consistent with studies of healthy aging showing Gln increases with age in humans and rodents (Hadel *et al*, 2013; Harris *et al*, 2014). With respect to schizophrenia, higher AC Gln was observed in antipsychotic naïve first episode patients and reduced in chronic medicated SZ (Theberge *et al*, 2002, 2003). In contrast, two other studies did not observe any AC Gln differences between medicated patients and controls (Tayoshi *et al*, 2009; Theberge *et al*, 2003). In a large study, Gln and Gln/Glu were elevated in participants with SZ (combination of first episode and chronic patients), and increased with aging across groups. These results are also consistent with a study reporting increased Gln/Glu with PCP, an NMDAR antagonist, in rodents (Iltis *et al*, 2009). Overall, lower Glu and higher Gln/Glu in schizophrenia indicates altered glutamatergic system possibly due to NMDAR hypofunction, but the exact mechanism remains to be determined.

Application of ASL to the study of cerebral blood flow alterations in SZ is relatively new. Previous studies in SZ utilized different acquisition and quantification methods for measuring rCBF; however, none of these studies examined SZ and age (Horn *et al*, 2009; Kindler *et al*, 2013; Ota *et al*, 2014; Pinkham *et al*, 2011; Scheef *et al*, 2010; Zhu *et al*, 2015). Two studies showed decreased GM rCBF in the anterior cingulate and frontal regions in SZ versus controls (Kindler *et al*, 2013; Zhu *et al*, 2015) but two other studies reporting no differences in medial frontal regions (Ota *et al*, 2014; Pinkham *et al*, 2011), similar to our results. In terms of WM rCBF, one did not observe any differences in SZ while another observed that WM rCBF decreased with age and faster in SZ (Pinkham *et al*, 2011; Wright *et al*, 2014). Similarly, this study found an aging effect of AC WM rCBF, which is also consistent with work showing rCBF decrease across life span (Lu *et al*, 2011).

In this study, a correlation between Glu and WM rCBF, and not AC GM rCBF, was observed. Examination of the tissue components within the voxel showed averages of 53% GM, 35% WM, and 12% CSF. We expected Glu to correlate with GM rCBF as Glu concentrations are higher in GM compared with WM (Michaelis *et al*, 1993). However, Glu in WM is highly detectable with MRS, and several studies highlight the important role of Glu neurotransmission in WM including signal propagation (Kukley *et al*, 2007). Also, this relationship was more pronounced in SZ, which converges nicely with reports of significant white matter alterations in SZ (Dias, 2012; Kochunov *et al*, 2014). We are unable to determine whether Glu in WM or GM is driving the correlation observed here. Future studies incorporating advanced MR techniques like short TE echo planar imaging EPSI (Ebel and Maudsley, 2003) are needed to differentiate white and gray matter Glu levels.

The potential impact of antipsychotic medication is inherent to the majority of schizophrenia research. The effect of antipsychotic medication on MRS measures remains

unknown. Some off medication studies reported higher levels of glutamatergic metabolites (de la Fuente-Sandoval *et al*, 2011, 2013a, 2013b, 2015; Kegeles *et al*, 2012; Kraguljac *et al*, 2013), whereas others reported reduced or no difference (Goto *et al*, 2012; Wood *et al*, 2008). However, higher levels of glutamatergic metabolites do not appear to be specific to the off medication state since several studies reported higher glutamatergic metabolites in medicated patients (Aoyama *et al*, 2011; Bustillo *et al*, 2014; Chang *et al*, 2007; Theberge *et al*, 2003) and in treatment resistant patients taking medication (Demjaha *et al*, 2014; Egerton *et al*, 2012; Mouchlianitis *et al*, 2016). Finally, a rodent MRS study showed no effect of 6 months antipsychotic treatment on Glu or Gln levels (Bustillo *et al*, 2006). Hence, the concept that glutamatergic metabolites are elevated in schizophrenia only in the off medication state and decline or normalize with antipsychotic treatment is not fully supported by empirical evidence.

Study limitations include voxel size and number, medication, macromolecules, and cross-sectional study. The voxel size translates to measures of Glu and rCBF from multiple regions within the frontal lobe and precludes conclusions about specific regions, as does the use of one voxel. Medications are a potential confound but statistical control of CPZ units indicated no effect. A limitation to utilizing CPZ units is that they only control for potency of dopamine D2 receptors. Nevertheless, studies are needed to determine the effects of medication on glutamatergic metabolites and rCBF in across the illness duration. Macromolecules are present in very short TE spectra. Similar to many other studies, the LCModel basis set of macromolecules was used for fitting the spectra. There are no studies that examined macromolecule levels between patients and controls. If differences exist, using the same MM basis set to fit both patients and controls could possibly impact the results. Studies comparing macromolecule levels in schizophrenia and control samples are needed. This study was cross-sectional and thus no conclusions can be made regarding the causal mechanisms underlying the reported observations; longitudinal studies are needed to examine the changes of Glu and rCBF with aging in SZ.

Overall, this is the first large scale study to show decreased Glu, increased Gln/Glu, and decreased WM rCBF in SZ from the anterior cingulate, suggesting an altered neurobiological state as determined by neurochemical and physiological metrics. These data do not support the notion that Glu declines more rapidly as function of age in SZ compared with controls; rather, these two groups have similar trajectories for Glu, Gln/Glu, and WM rCBF in this region.

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The authors declare no conflict of interest.

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