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Increased hippocampal blood volume and normal blood flow in schizophrenia

Pratik Talati^{*,a}, Swati Rane^{*,b}, Jack Skinner^b, John Gore^b, and Stephan Heckers^a

^aVanderbilt Brain Institute, Department of Psychiatry, Vanderbilt University, Nashville, TN, 37212 USA

^bInstitute of Imaging Science, Department of Radiology and Radiological Sciences, Vanderbilt University, Nashville, TN, 37232 USA

Abstract

Neuroimaging studies have provided compelling evidence for abnormal hippocampal activity in schizophrenia. Most studies made inferences about baseline hippocampal activity using a single hemodynamic parameter (e.g., blood volume or blood flow). Here we studied several hemodynamic measures in the same cohort to test the hypothesis of increased hippocampal activity in schizophrenia. We used dynamic susceptibility contrast- (DSC-) magnetic resonance imaging to assess blood volume, blood flow, and mean transit time in the hippocampus of 15 patients with chronic schizophrenia and 15 healthy controls. Left and right hippocampal measurements were combined for absolute measures of cerebral blood volume (CBV), blood flow (CBF), and mean transit time (MTT). We found significantly increased hippocampal CBV, but normal CBF and MTT, in schizophrenia. The uncoupling of CBV and CBF could be due to several factors, including antipsychotic medication, loss of cerebral perfusion pressure, or angiogenesis. Further studies need to incorporate several complementary imaging modalities to better characterize hippocampal dysfunction in schizophrenia.

Keywords

Dynamic susceptibility contrast; DSC-MRI; Gadolinium; Mean transit time; CBV; CBF

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^{*}Co-corresponding authors: Pratik Talati, Vanderbilt University, Department of Psychiatry, 3057 VPH, 1601 23rd Ave South, Nashville, TN 37212, pratik.talati@vanderbilt.edu, 615-875-5897; Swati Rane, Vanderbilt University, Institute of Imaging Science, AA3101 MCN, 1161 21st Ave South, Nashville, TN 37232, swati.rane@vanderbilt.edu, 615-322-7149.

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Contributors

Pratik Talati and Swati Rane both contributed equally to the data collection, analysis, and manuscript preparation. Jack Skinner contributed with data analysis and manuscript revisions. Stephan Heckers and John Gore helped with project inception, funding, data interpretation, and manuscript revisions.

Conflict of interest

The authors disclose no conflict of interest.

1. Introduction

The hippocampus is abnormal in schizophrenia (Heckers and Konradi, 2010). Studies of the underlying cellular and molecular mechanisms have illustrated *N*-methyl-D-aspartate (NMDA) receptor hypofunction (Olney et al., 1999) and reduced gamma-aminobutyric acid- (GABA-) ergic interneuron density (Benes et al., 1998), especially in fast-spiking, parvalbumin-containing cells (Zhang and Reynolds, 2002; Konradi et al., 2011). These molecular changes may lead to excitation-inhibition imbalances (Lisman et al., 2008; Heckers and Konradi, 2014), which can be indirectly assessed through perfusion imaging methods. Initial positron emission tomography (PET) and single photon emission computed tomography (SPECT) studies measured cerebral blood flow (CBF) or glucose metabolism (in the case of PET) or volume (CBV). These studies failed to achieve a general consensus: some reported increases in baseline hippocampal activity (Friston et al., 1992; Heckers et al., 1998; Malaspina et al., 2004), while others illustrated decreases (Buchsbaum et al., 1992; Tamminga et al., 1992; Nordahl et al., 1996) or no changes (Vita et al., 1995). Due to radiation exposure and poor spatial resolution, these methods are now used less frequently than comparable magnetic resonance imaging (MRI) methods (Small et al., 2011).

Recently implemented MRI-based methods require an endogenous (blood water) or exogenous (paramagnetic) contrast agent to generate CBV or CBF maps. Arterial spin labeling uses blood water magnetization as an endogenous contrast to analyze a single hemodynamic parameter, i.e., CBF, in the brain. This method has provided mixed results, finding increases (Pinkham et al., 2011), decreases (Scheef et al., 2010; Walther et al., 2011; Kindler et al., 2015), or no changes (Horn et al., 2009; Ota et al., 2014) in medial temporal lobe CBF in schizophrenia. More recently, contrast-enhanced, high resolution (submillimeter) steady-state imaging has been used to investigate CBV changes in schizophrenia. Though few in number, these initial studies support increased hippocampal Cornu Ammonis 1 (CA1) CBV (Schobel et al., 2009; Schobel et al., 2013; Talati et al., 2014a). Importantly, this method also characterizes a single hemodynamic parameter, i.e., CBV, to make inferences about basal metabolism. Dynamic susceptibility contrast- (DSC-) MRI may be able to resolve these mixed findings of hippocampal activity across different modalities due to its ability to assess *several* hemodynamic parameters, including CBV, CBF, and mean transit time (MTT) (Perkio et al., 2002).

Even though DSC-MRI has been used extensively to investigate metabolism and perfusion abnormalities in neurological disorders such as tumors and strokes, very few studies have implemented this technique to study psychiatric illnesses including schizophrenia (Renshaw et al., 1997). As recently reviewed by Théberge in 2008, only five articles have been published using this method in schizophrenia research (Théberge, 2008). Since the review, there have been two additional studies of bolus-tracking perfusion imaging with a paramagnetic agent in schizophrenia (Bellani et al., 2011; Peruzzo et al., 2011). Together, these studies illustrate several findings in schizophrenia: low and inverse hemispheric CBV as indirectly measured through contrast enhancement (CE) (Brambilla et al., 2007); increased CBV in the cerebellum (Loeber et al., 1999), caudate, and occipital cortex (Cohen et al., 1995); no alterations in CBV, CBF, or MTT in the cerebrum or cerebellum (Bellani et al., 2011); decreased frontal cortex CBV and CBF only when using a best predictor model

containing clinical state, age, and length of illness (Peruzzo et al., 2011); variable time-to-peak (TTP) in the caudate (Fabene et al., 2007); and increased perfusion in the prefrontal cortex, temporal lobe, and posterior parietal cortices after dopamine receptor D1 agonist administration (Mu et al., 2007). Importantly, none of these studies used a region of interest-based analysis to report hemodynamic properties in the hippocampus in schizophrenia, even though functional abnormalities have been reported in this medial temporal lobe structure.

In this study, we used DSC-MRI to specifically study hippocampal perfusion properties (CBV, CBF, and MTT) in 15 patients with chronic schizophrenia and matched controls. Based on a previous report on this cohort using steady-state CBV imaging (Talati et al., 2014a), we hypothesized increased hippocampal perfusion, as measured by CBV and CBF.

2. Methods

2.1. Participants

Participants comprised 15 patients with schizophrenia or schizoaffective disorder (age range: 20-54 years) and 15 matched healthy controls (age range: 22-53 years), who provided informed consent in a manner approved by the Vanderbilt Institutional Board. Both groups were matched across several demographic variables, including age, race, and gender (Table 1). Subjects were recruited from the Vanderbilt Psychotic Disorders Program or the local community; they were paid for their participation. We used the Structured Clinical Interview for DSM-IV Axis I disorders [SCID (First, November 2002)] to establish all diagnoses and the Positive and Negative Syndrome Scale (Kay et al., 1987) to assess the clinical status of the patients. Most of the patients (12 of 15) were treated with antipsychotic medication, and their chlorpromazine-equivalent dosages (Gardner et al., 2010) are listed in Table 1. History of major neurological or medical illness was an exclusion criterion. Since nephrogenic systemic fibrosis is a rare adverse effect of gadolinium-containing contrast, we required a normal serum creatinine value for study eligibility and confirmed normal values again after study completion.

2.2. Dynamic susceptibility contrast imaging

Imaging was performed using a Phillips 3T Achieva scanner with an eight-channel SENSE head coil. T2*-weighted echo planar images were acquired perpendicular to the long axis of the hippocampus for accurate segmentation using the following sequence parameters: repetition time (TR) = 1600 ms, echo time (TE) = 54 ms, Field-of-View (FOV) = 240×240 mm², spatial resolution: 1.5 mm³ isotropic, slices = 15, dynamics = 120. Fourteen dynamic scans were carried out before gadopentetate dimeglumine (Magnevist®, Bayer Schering Pharma, Germany, 0.1 mmol/kg) was injected at a constant rate of 5 ml/s through an 18G intravenous catheter in the antecubital vein via an MRI-compatible power injector (Medrad®, PA, USA). The bolus of contrast was immediately followed by a 40-ml saline flush at the same rate. The intravenous catheter was removed after the completion of the scan. To allow for accurate segmentation of the hippocampal formation (Moreno et al., 2007), high-resolution T1-weighted pre-contrast images were also acquired perpendicular to the long axis of the hippocampus using the following scan parameters: TR = 20 ms, TE = 3.41 ms, FOV = 256×256 mm², slices = 15.

2.3. Analysis

2.3.1. CBV, CBF, and MTT calculations—AFNI (3dvolreg) (Cox, 1996) was used to correct for subject motion during the dynamic scans, and all images were registered to the first dynamic scan. First, voxel-wise signal contrast variations due to injection of gadolinium were converted to a concentration-time curve using Equation 1 (Ostergaard, 2004):

$$\Delta R_2^*(t) = \frac{-\log\left(\frac{S(t)}{S_0}\right)}{TE} \quad (\text{Eq. 1})$$

where R_2^* is the change in transverse relaxation rate (i.e., $1/T_2^*$); TE is the echo time; $S(t)$ is the signal intensity at a time t ; and S_0 is the baseline signal intensity at a voxel, calculated as the mean signal intensity at that voxel over the first 10 dynamic scans before contrast administration. Voxels located near the M1 segment of the middle cerebral artery were used to evaluate the arterial input function (AIF). Care was taken to place the voxels adjacent to the vessel to avoid susceptibility effects and signal cutoff as outlined by (van Osch et al., 2005). CBV, CBF, and MTT were calculated using singular value decomposition (SVD) with block circulant matrices (Ostergaard et al., 1996a; Ostergaard et al., 1996b). The threshold for the diagonal matrix generated using SVD was set to 0.15 to reduce oscillations of the derived tissue residue function. This threshold was chosen based on the high resolution and low signal-to-noise ratio of the images ($\text{SNR} = 7.60 \pm 2.02$). Regional CBF value was calculated as the peak value of the deconvolved tissue impulse response. Regional CBV was the ratio of the area under the tissue R_2^* curve to the area under the AIF R_2^* curve. Mean transit time (MTT) was calculated using Equation 2:

$$MTT = CBV / CBF \quad (\text{Eq. 2})$$

Finally, a normal appearing white matter region of interest was chosen in the parietal region for each subject. Assuming a white matter CBF of 22 ml/100 g/min and a CBV of 2 ml/100 g (Ostergaard et al., 1996a), the regional values were scaled to obtain absolute CBF and CBV measures. Matlab (version 7.13.0.564, The MathWorks Inc, Natick, MA) was used to generate an in-house script to obtain the CBV, CBF, and MTT values.

2.3.2. Hippocampal segmentation—The T1-weighted pre-contrast image was used for blind, manual segmentation of the hippocampal formation by one rater (PT) and verified by another rater (SR). If there were any discrepancies, the region of interest (ROI) was drawn by both raters until a consensus was reached. Manual segmentation of each subject's hippocampus generated 15 hippocampal ROIs for each hemisphere for a total of 30 hippocampal ROIs (15 left and 15 right). Hippocampal ROIs were down-sampled to reach the same spatial resolution of the T2*-weighted images.

2.3.3. Statistics—CBF, CBV, and MTT values across all posterior hippocampal slices were averaged for each hemisphere for each subject. This method produced the following six values for each subject: left and right hippocampal CBV, left and right hippocampal CBF, and left and right hippocampal MTT.

Because there was no *a priori* hypothesis about hemispheric lateralization, we performed an exploratory analysis testing for hemisphere effects. A repeated-measures analysis of variance (ANOVA) with hemisphere as a within-subject factor and group as a between-subject factor illustrated no main effect of hemisphere and no hemisphere by diagnosis interaction for CBV ($F(1,28) = 1.83, p = 0.19$; $F(1,28) = 0.62, p = 0.44$, respectively), CBF ($F(1,28) = 2.17, p = 0.15$; $F(1,28) = 0.11, p = 0.74$, respectively), and MTT ($F(1,28) = 0.76, p = 0.39$; $F(1,28) = 0.56, p = 0.46$, respectively). Therefore, the primary analysis for each hemodynamic parameter (CBV, CBF, and MTT) was a between-group independent samples *t*-test after collapsing across hemisphere (average left/right). Because groups were well matched, age, gender, and race were not included as covariates in the analysis. Correlation analyses are reported using Pearson's correlation. Statistical analyses were performed using The Statistical Package for Social Sciences software (SPSS version 20, IBM Corporation, Armonk, NY: <http://www.spss.com>).

3. Results

Fig. 1 depicts the R_2^* changes before, during, and after gadolinium-contrast administration. Injection with a gadolinium bolus causes predominant R_2^* shortening, resulting in a loss of signal. As the contrast agent recirculates, the bolus diffuses and equilibrates with the blood in the microvasculature, allowing the tissue R_2^* to recover to nearly its pre-contrast value (shown in Fig. 2).

3.1. Hemodynamic imaging

Whole brain CBV-, CBF-, and MTT-weighted maps were generated for each subject (see Fig. 3) to extract hippocampal CBV, CBF, and MTT values. For healthy controls, hippocampal CBV (units = ml blood/100 g parenchyma) ranged from 2.69 to 5.32. Patients had a wider hippocampal CBV range (2.80 to 8.48). Hippocampal CBF (units = ml blood/100 g parenchyma per min) ranged from 27.75 to 76.71 in healthy controls and from 35.39 to 84.65 in patients. Finally, healthy control hippocampal MTT (units = seconds) ranged from 3.28 to 7.30 and patient hippocampal MTT ranged from 4.09 to 8.14. These values are similar to those previously published for medial temporal lobe CBF (Li et al., 2013) and CBV (Schobel et al., 2009; Talati et al., 2014b) and comparable to contrast-enhanced steady-state CBV values in the same cohort (Talati et al., 2014a).

We first tested the hypothesis of increased hippocampal metabolism, as measured by CBV and CBF, in schizophrenia. Hippocampal CBV (mean $\pm$ SD) was increased in patients with schizophrenia compared with healthy controls (4.57 ± 1.41 vs. 3.79 ± 0.86 , one-tailed *t*-test, $t(28) = 1.83, p = 0.039$, Cohen's $d = 0.67$, Fig. 4A), when averaged across hemispheres. However, there were no group differences in CBF (one-tailed *t*-test, $t(28) = -0.63, p = 0.28, d = 0.21$, Fig. 4B). We then investigated whether hippocampal MTT was altered in schizophrenia and found no differences (two-tailed *t*-test, $t(28) = -1.23, p = 0.22, d = 0.46$), as illustrated in Fig. 4C.

We explored whether increased CBV in schizophrenia correlated with duration of illness, dose of antipsychotic medication, or psychopathology as assessed by the Positive and Negative Syndrome Scale (PANSS). Hippocampal CBV did not correlate with duration of

illness ($r = -0.28$, $p = 0.31$), positive symptoms ($r = -0.13$, $p = 0.65$), negative symptoms ($r = -0.43$, $p = 0.11$), or general psychopathology ($r = 0.001$, $p = 1.00$) in the patient group. Three patients' chlorpromazine (CPZ) equivalent dosages could not be calculated because there was either no chlorpromazine equivalent dose ($n=1$) or they were not taking any medication at the time of the study ($n=2$). For the remaining 12 patients, antipsychotic medication dose did not correlate with hippocampal CBV ($r = 0.43$, $p = 0.16$).

4. Discussion

This is the first study to use DSC-MRI to study hippocampal perfusion in schizophrenia. We report increased hippocampal CBV in chronic schizophrenia, similar to our previous study using steady-state imaging in the same cohort (Talati et al., 2014a). However, we did not find increased hippocampal CBF or MTT in schizophrenia.

Because of the well-established relationship between CBV and CBF (Grubb et al., 1974), many studies report CBV or CBF changes as a marker of basal activity. Here we report a novel finding in schizophrenia: increased hippocampal blood volume in the context of normal hippocampal blood flow. Our finding of increased hippocampal CBV is consistent with other gadolinium-enhanced studies (Schobel et al., 2009; Schobel et al., 2013). We did not observe increased hippocampal CBF using DSC-MRI, similar to what has been reported using arterial spin labeling (Horn et al., 2009; Ota et al., 2014) and nuclear medicine techniques (Vita et al., 1995). Even with the large variance in our dataset, the calculated effect sizes are larger for CBV ($d = 0.67$) than CBF ($d = 0.21$), suggesting that CBF changes may be more difficult to detect.

There might be several reasons for the finding of increased CBV in the context of normal CBF. First, previous studies have shown that antipsychotic medications do not affect hippocampal CBV (Schobel et al., 2009; Schobel et al., 2013; Talati et al., 2014a) but that they lower CBF in many brain regions (Miller et al., 1997; Novak et al., 2005; Ertugrul et al., 2009), including the hippocampus (Medoff et al., 2001; Lahti et al., 2003). Second, hippocampal excitation-inhibition imbalances (Lisman et al., 2008; Heckers and Konradi, 2014) in the prodromal or early stage of psychosis may lead to increased CBF to meet greater hippocampal metabolic demand. Increased CBF demands are mediated by increases in CBV via complex myogenic, chemical, neuronal, or metabolic autoregulatory mechanisms. Such CBV-CBF coupling occurs only when cerebral perfusion pressure remains unchanged. Therefore, a lack of CBF increase is likely due to a reduction in the cerebral perfusion pressure (Heilbrun et al., 1972; Chan et al., 1992). Finally, instead of microvascular dilation, increased CBV can be secondary to angiogenesis (Swain et al., 2003), which could be assessed via hemodynamic response imaging (i.e., carbogen challenge (95% O₂+5% CO₂) that vasodilates all functional blood vessels and a hypercapnic challenge (95% air+5% CO₂) that only vasodilates mature blood vessels (Ben Bashat et al., 2012).

A major goal in schizophrenia research is to link brain function with clinical features of the disease. Many perfusion imaging studies have quantified a single hemodynamic index (e.g., CBV or CBF) and correlated abnormal perfusion with psychopathology. For example,

increased medial temporal lobe CBF has been associated with more severe general psychopathology (Friston et al., 1992) and positive symptoms (Liddle et al., 1992; Lahti et al., 2006), while increased hippocampal CBV has been associated with positive and negative symptoms (Schobel et al., 2009; for review, see Tregellas, 2014). Furthermore, increased CBF in the superior temporal gyrus predicted a reduction of auditory hallucinations after treatment with transcranial magnetic stimulation (Homan et al., 2012), suggesting that decreased perfusion in this region may predict which patients are likely to respond to this treatment. However, not all studies using CBV or CBF have supported correlations between brain function and positive/negative symptoms (Kawasaki et al., 1992; Talati et al., 2014a).

There are several assumptions that commonly underlie the DSC-MRI data analysis (Calamante et al., 2002). First, there is an assumption of linearity between tissue contrast agent concentration and changes in the R_2^* relaxation rate (Equation 1). This assumption has been validated (Boxerman et al., 1995; Simonsen et al., 1999) and likely holds true. Second, the arterial input function is presumed to represent the exact input to the tissue of interest (in this case, the hippocampus). The hippocampus has a differential blood supply, with the anterior region supplied by anterior hippocampal branch of the posterior cerebral artery (PCA) and anterior choroidal artery and the posterior region supplied by the middle and posterior hippocampal branches of the PCA (Huang and Okudera, 1997). In practice, however, the M1 segment of the MCA is used and has generated reliable CBF values when compared with PET studies (Zaro-Weber et al., 2012). Furthermore, we manually selected the AIF to avoid partial volume effects that can lead to under- or overestimation of the contrast agent concentration (van Osch et al., 2005). Other assumptions include constant capillary hematocrit and vascular permeability across brain regions and between subjects, which are not necessarily true.

The ideal imaging experiment to interrogate hippocampal hyperactivity would utilize a reliable, non-invasive imaging technique to longitudinally follow high-risk individuals who eventually convert to psychosis. Arterial spin labeling (ASL) is one such non-invasive method that has been shown to be reliable within and between scanning sessions (Parkes et al., 2004; Petersen et al., 2010; Xu et al., 2010; Gevers et al., 2011; Donahue et al., 2014). Even though ASL is gaining popularity due to increased signal-to-noise ratio at higher magnetic fields (e.g. 3 T) and ease of implementation, there are some drawbacks to this method: the reproducibility is quite variable in different brain regions (Wu et al., 2014), it has worse spatial and temporal resolution than DSC-MRI (Wintermark et al., 2005), the validity of the reported values varies depending on disease conditions (Tanaka et al., 2011; Zaharchuk et al., 2012; Choi et al., 2013; Nael et al., 2013; Jiang et al., 2014; White et al., 2014), and it is unclear how antipsychotic medication affects CBF values. Newer variants of arterial spin labeling acquire data over multiple post-labeling delays to assess several hemodynamic parameters (e.g. CBV and CBF) and have been shown to correlate with DSC-MRI (Wang et al., 2013). However, the spatial resolution still may lead to some challenges in interpreting CBF and CBV values in small structures like the hippocampus.

This study has a few limitations. The sample size is small but consistent with other CBV imaging studies of schizophrenia patients (Schobel et al., 2009; Talati et al., 2014a). Most of our patients were treated with antipsychotic medication, which can affect hippocampal

hemodynamic parameters such as CBF (see above). Furthermore, this method does not provide enough spatial resolution for subfield-specific investigation. While the in-plane resolution is 1.5 mm isotropic, the actual in-plane resolution is closer to 2 mm when considering the point-spread function degradation due to the paramagnetic bolus passage (Wintermark et al., 2005). Therefore, subfield-specific testing (Small et al., 2011) was not feasible in this dataset.

In conclusion, we report increased hippocampal CBV, but not CBF, in chronic schizophrenia. Future studies need to incorporate several complementary imaging modalities to better characterize hippocampal dysfunction in schizophrenia.

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- Hippocampal activity is abnormal in schizophrenia
- CBV or CBF is typically reported to indirectly assess hippocampal activity
- We used DSC-MRI to assess several hippocampal hemodynamic parameters
- Hippocampal CBV, but not CBF or MTT, is increased in schizophrenia
- Complementary imaging methods are needed to best understand hippocampal dysfunction

Fig. 1.

T1- and T2*-weighted dynamic scans in radiological orientation for a representative control subject with contrast-enhanced imaging.

A: T1-weighted structural image. B: T2*-weighted dynamic scan before contrast administration. C: Loss of signal intensity on T2*-weighted images during contrast administration. D: Recovery of signal intensity after contrast administration.

Fig. 2.

R_2^* signal recovery after gadolinium bolus passage in two different brain regions of a control subject

After a gadolinium bolus, R_2^* signal changes are evident across 120 dynamic scans (192 s) in select voxels near the M1 segment of the middle cerebral artery for the arterial input function (AIF, open circles connected by a solid line) and gray matter tissue (filled circles connected by a dashed line). The figure inset indicates the tissue response function obtained after deconvolution.

Fig. 3.

Hemodynamic maps for a representative control subject in radiological orientation

A: T2*-weighted dynamic susceptibility contrast (DSC) dynamic scan. B-D: cerebral blood volume-(CBV-), cerebral blood flow-(CBF-), and mean transit time- (MTT-)weighted maps, respectively. Color bar denotes values for each hemodynamic map, with lighter colors corresponding to larger values.

Fig. 4.

Increased hippocampal blood volume, but not flow, in schizophrenia

Hemodynamic parameters (mean $\pm$ SD) for healthy controls and patients with schizophrenia generated from dynamic susceptibility contrast-(DSC)-MRI for the hippocampus. These values are averaged across hemisphere and slice along the long axis of the hippocampus. Fig. 4A illustrates increased hippocampal cerebral blood volume (CBV) in schizophrenia (one-tailed t -test, $t(28) = 1.831$, $p = 0.039$). Fig. 4B illustrates no group differences in hippocampal cerebral blood flow (CBF, one-tailed t -test, $t(28) = -0.629$, $p = 0.28$), and Fig. 4C illustrates no difference in hippocampal mean transit time (MTT) in schizophrenia (two-tailed t -test, $t(28) = -1.230$, $p = 0.22$). * $p < 0.05$.

Table 1

Subject demographics in individuals with schizophrenia and healthy controls

Groups are matched on age, gender, race, and subject and parental education. Values are reported as mean $\pm$ SD. The chlorpromazine (CPZ) equivalent doses could not be calculated for two subjects who were off medication at the time of the study and for one subject who was taking asenapine. PANSS denotes the Positive and Negative Syndrome Scale.

	Controls (<i>n</i> = 15)	Schizophrenia (<i>n</i> = 15)	Statistic	<i>p</i>-value
Age (years)	34.27 $\pm$ 9.06	36.20 $\pm$ 12.59	<i>t</i> (25.43) = -0.483	0.63
Males	10	10	X(1) = 0	1.00
Race (W/B)	11/4	10/5	X(1) = 0.159	0.69
Subject edu. (years)	15.53 $\pm$ 2.77	14.20 $\pm$ 2.51	<i>t</i> (28) = 1.38	0.18
Avg. parental edu. (years)	14.40 $\pm$ 1.91	14.19 $\pm$ 2.03	<i>t</i> (28) = 0.30	0.77
Duration of Illness (years)		10.55 $\pm$ 8.86		
CPZ equivalent (mg/day)		352.50 $\pm$ 160.35		
PANSS		Positive: 15.47 $\pm$ 7.10		
		Negative: 17.47 $\pm$ 7.22		
		General: 28.07 $\pm$ 8.56		