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QUANTITATIVE ANALYSIS OF GLUTAMATE TRANSPORTER mRNA EXPRESSION IN PREFRONTAL AND PRIMARY VISUAL CORTEX IN NORMAL AND SCHIZOPHRENIC BRAIN

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Abstract—Abnormalities of the glutamatergic system in schizophrenia have been identified in numerous studies, but little is known about the role of glutamate transporters and their messenger RNA (mRNA) expression. In addition, the abundances of the two major isoforms of human excitatory amino acid transporter 2 (*EAAT2*) or its rat ortholog, glutamate transporter 1, have never been compared in a quantitative manner. Using quantitative reverse transcription–polymerase chain reaction, we established that the expression of the *EAAT1, EAAT2a, EAAT2b,* and *EAAT3* transcripts was not different in the dorsolateral prefrontal and primary visual cortices of persons with schizophrenia relative to matched controls. *EAAT2a* expression was about 25-fold and 10-fold higher than *EAAT2b* in human and rat brain, respectively. The data provided no evidence of an effect of antipsychotic medications on the mRNA expression of the glutamate transporters. However, because most of the schizophrenic subjects in the cohort had been treated with antipsychotics for many years, it is still possible that changes in transporter expression were masked by medication effects.

Key words: quantitative PCR, EAAT2, GLT1, gene expression, antipsychotic, RT-PCR.

Glutamatergic transmission plays a critical role in a variety of nervous system functions, including learning and memory (Bliss and Collingridge, 1993); excessive extracellular glutamate, however, causes neurotoxicity (Choi, 1994; Farber et al., 2002; Olney et al., 1999). The pivotal role of glutamatergic neurotransmission suggests that the regulation of glutamate levels in the central nervous system plays an important role in normal and abnormal functions of the brain.

Five different high-affinity glutamate transporters maintain glutamate levels within a narrow range necessary for synaptic transmission without causing excitotoxicity (Danbolt, 2001; Robinson, 1998). The excitatory amino acid transporter 2 (*EAAT2*) (glutamate transporter 1, *Glt1*, in rodents) is highly expressed in the forebrain (Pines et al., 1992) and is responsible for over 90% of glutamate uptake (Haugeto et al., 1996; Tanaka et al., 1997). The *EAAT2* gene is alternatively spliced; the major messenger RNA (mRNA) variants—*EAAT2a* and *EAAT2b*—encode functional transporters that differ in their carboxyl termini. *EAAT2a* is the most abundant isoform and is mainly found in astrocytes (Chaudhry et al., 1995; Lehre et al., 1995; Rothstein et al., 1994), although its RNA (Berger and Hediger, 1998; Schmitt et al., 1996; Torp et al., 1997) and protein (Chen et al., 2004) are also present in neurons. The *EAAT2b* isoform was regarded as the neuronal isoform (Chen et al., 2002; Kugler and Schmitt, 2003; Schmitt et al., 2002), but has been detected in astrocytes as well (Chen et al., 2002; Reye et al., 2002; Sullivan et al., 2004). *EAAT2a* and *EAAT2b* appear to be differentially regulated in various pathological conditions such as amyotrophic lateral sclerosis (ALS), where a significant decrease in *EAAT2a* expression is accompanied by an increase in *EAAT2b* expression (Maragakis et al., 2004). Another transporter that is expressed in astrocytes, *EAAT1* (glutamate and aspartate transporter, *Glast*, in rodents), is found mainly in the cerebellum, as well as in the heart, skeletal muscle, and placenta (Schmitt et al., 1997; Storck et al., 1992). *EAAT3* (excitatory amino acid carrier 1, *Eaac1*, in rodents) is a neuronal transporter that is abundant in the cerebral cortex, hippocampus, red nucleus, substantia nigra, and caudate-putamen, and is also present in the intestine, kidney, liver, and heart (Kanai and Hediger, 1992; Rothstein et al., 1994; Shashidharan et al., 1994). Expression of the other two *EAATs* is very restricted: *EAAT4* is found predominantly in cerebellar Purkinje cells (Fairman et al., 1995) and *EAAT5* is expressed primarily in the retina (Arriza et al., 1997).

Multiple lines of evidence suggest that disturbances of the glutamatergic system are involved in the etiology of schizophrenia (SZ) (Goff and Coyle, 2001; Gluck et al., 2002; Haroutunian et al., 2003; Tsai et al., 1998). The expression of glutamate receptor subunits and other mark-

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Abbreviations used: ALS, amyotrophic lateral sclerosis; ANCOVA, analysis of covariance; C_t, threshold cycle; DLPFC, dorsolateral prefrontal cortex; *Eaac1*, excitatory amino acid carrier 1; *EAAT*, excitatory amino acid transporter; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase; *Glast*, glutamate and aspartate transporter; *Glt1*, glutamate transporter 1; GM, geometric mean; mRNA, messenger RNA; NCs, normal controls; PMI, postmortem interval; PVC, primary visual cortex; qPCR, quantitative polymerase chain reaction; rRNA, ribosomal RNA; RT, reverse transcription; SZ, schizophrenia; SZs, schizophrenic subjects; SZs-off, schizophrenic subjects off medication at least 4 weeks; VA, Veterans Affairs.

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ers of glutamatergic transmission is altered in postmortem brain tissue from individuals with SZ (Meador-Woodruff and Healy, 2000). There is also some evidence of altered glutamate transporter expression in individuals with SZ (Matute et al., 2005; McCullumsmith and Meador-Woodruff, 2002; Ohnuma et al., 1998, 2000; Smith et al., 2001).

Altered *EAAT* expression was detected in the thalamus (Smith et al., 2001) and striatum (McCullumsmith and Meador-Woodruff, 2002) in the subjects with SZ from the cohort used in the present study. Because of the reciprocal connections between these structures and cortex, the objective of the present study was to compare the expression of the *EAAT1*, *EAAT2a*, *EAAT2b*, and *EAAT3* mRNAs in the dorsolateral prefrontal cortex (DLPFC) and primary visual cortex (PVC) of elderly patients with SZ with that of matched normal controls (NCs) using reliable quantitative methodology. The DLPFC was selected because neuroimaging and neuroanatomical studies have consistently provided evidence of abnormalities in subjects with SZ in this area (Bunney and Bunney, 2000; Volk and Lewis, 2002; Weinberger et al., 1986). In contrast, evidence of dysregulation in the occipital cortex in SZ is limited, and therefore, the PVC was included in the study to test the regional specificity of any changes in *EAAT* expression. However, a few imaging and morphological studies reported abnormalities in the PVC in SZ (Mitelman et al., 2003; Selemon et al., 1995). In addition, by examining *EAAT* expression in the two brain regions, we sought to gain a better understanding of *EAAT* gene expression heterogeneity across functionally different brain regions. Because *EAAT4* and *EAAT5* are not expressed at substantial levels in the cerebral cortex, these transporters were not examined here. We also compared the abundances of the two major functional isoforms of *EAAT2* in the human and rat cortex.

EXPERIMENTAL PROCEDURES

Human postmortem tissue

Frozen postmortem brain tissue from the DLPFC (Brodmann area 46) and PVC (Brodmann area 17) of subjects diagnosed with schizophrenia (SZs) ($n=34$ for DLPFC, $n=36$ for PVC) by *Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition* (DSM-IV) criteria and NCs ($n=26$) was obtained from the Mount Sinai/Bronx Veterans Affairs (VA) Medical Center Department of Psychiatry Brain Bank. The DLPFC was dissected according to previously defined criteria (Rajkowska and Goldman-Rakic, 1995), and the PVC was defined as the region containing the band of Gennari in the coronal section from approximately 2 cm rostral to the occipital pole. Gray matter was carefully dissected from these regions. The sex distribution, mean age, postmortem interval (PMI), and tissue pH of the cohort used in this study are shown in Table 1. There were no significant differences between diagnostic cohorts with respect to age and sample pH for either cortical region; however, PMIs were significantly longer in SZs in both regions ($t\geq2.33$, $df=60$, $P\leq0.023$). Also, there were significant sex differences (more males in SZs, more females in NCs) in both cortical regions ($\chi^2\geq4.24$, $df=1$, $P\leq0.035$).

The institutional review boards of Pilgrim Psychiatric Center, Mount Sinai School of Medicine, and the Bronx VA Medical Center approved all assessment and postmortem procedures. Written informed consent for autopsy and use of brain tissue in research was obtained from the next of kin. All patients had thorough neuropathologic characterization to rule out discernable neuropathologies such as Alzheimer's disease and multi-infarct dementia (Purohit et al., 1998). NCs had no history of any psychiatric or neurological disorders and no identifiable neuropathologic lesions. Of the SZs, 13 from the DLPFC and 15 from the PVC were off antipsychotic medications for at least four weeks before death (SZs-off) (range off antipsychotic drugs: 1 month to 7 years).

Table 1. Demographic characteristics of the cohort

	DLPFC		PVC	
	NCs	SZs	NCs	SZs
Sex, M/F	9/17	21/13*	9/17	22/14*
pH	6.48±0.05	6.52±0.05	6.48±0.05	6.51±0.05
age	81.2±2.2	76.1±1.8	81.2±2.2	76.1±1.8
PMI	507.9±82.3	800.2±78.9*	507.9±82.3	773.6±76.7*

Values are expressed as mean±SEM.
* $P<0.05$ between SZs and NCs.

RNA isolation and reverse transcription (RT)

Total RNA was isolated from 50 mg of human or rat brain tissue by the guanidinium isothiocyanate method, using the ToTALLY RNA kit (Ambion, Austin, TX, USA). To remove genomic DNA contamination, the isolated RNA samples were then treated with 40 U DNase I (Ambion) in the presence of 120 units of RNaseOUT (Invitrogen, Carlsbad, CA, USA) for 1 h at 37 °C. RNA concentration was determined by absorption at 260 nm, and the 260/280 nm absorption ratios of the samples were >2.1. The quality of the total RNA was also assessed on an ethidium bromide-stained agarose gel. Only samples with clearly defined 18S and 28S ribosomal RNA peaks were used in the study. RNA yields were typically between 15 and 30 μg and did not differ between NCs and SZs. The ThermoScript RT System (Invitrogen) was used for the RT reactions with random hexamers and approximately 2 μg of total RNA from each individual. The cDNA was diluted 1:25 and 5 μL was used in each 25 μL PCR reaction.

Real-time PCR

EAAT mRNA expression was measured by quantitative polymerase chain reaction (qPCR) with gene-specific fluorogenic TaqMan probes. Pre-designed TaqMan Gene Expression assays were purchased from Applied Biosystems (Foster City, CA, USA) for *EAAT1*, *EAAT2*, *EAAT3*; custom-designed assays were used for *EAAT2b* and β-actin. The probe and primer sets for 18S ribosomal RNA (rRNA) and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) were TaqMan control reagents. Probe and primer sequences for custom assays and assay ID numbers for pre-designed assays are listed in Table 2. The 20× TaqMan assay mixes were used at a final concentration of 0.5× (18S rRNA was used at 0.25×). The reaction mixes included one unit AmpliTaq Gold DNA polymerase, 1× PCR buffer II, 4 mM magnesium chloride, 0.25 unit AmpErase uracil-*N*-glycosylase, 200 μM each of dATP, dCTP, dGTP, 400 μM of dUTP (Applied Biosystems), and the reference dye ROX at a 0.7× final concentration (Invitrogen). Real-time PCR was performed in an ABI 7700 Prism Sequence Detector (Applied Biosystems). The thermal cycling program consisted of 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The reactions were quantified by selecting the amplification cycle when the PCR product of interest was first detected (threshold cycle, C_t). Tests of assay linearity were conducted for all real-time PCR assays using serial dilutions of a pooled sample as previously described