

# Cortical Glutamatergic Markers in Schizophrenia

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Post-mortem studies have yet to produce consistent findings on cortical glutamatergic markers in schizophrenia; therefore, it is not possible to fully understand the role of abnormal glutamatergic function in the pathology of the disorder. To better understand the changes in cortical glutamatergic markers in schizophrenia, we measured the binding of radioligands to the ionotropic glutamate receptors (*N*-methyl-D-aspartate, [<sup>3</sup>H]CGP39653, [<sup>3</sup>H]MK-801), amino-3-hydroxy-5-methyl-4-isoxazole ([<sup>3</sup>H]AMPA), kainate ([<sup>3</sup>H]kainate), and the high-affinity glutamate uptake site ([<sup>3</sup>H]aspartate) using *in situ* radioligand binding with autoradiography and levels of mRNA for kainate receptors using *in situ* hybridization in the dorsolateral prefrontal cortex from 20 subjects with schizophrenia and 20 controls matched for age and sex. Levels of [<sup>3</sup>H]kainate binding were significantly decreased in cortical laminae I–II ( $p = 0.01$ ), III–IV ( $p < 0.05$ ), and V–VI ( $p < 0.01$ ) from subjects with schizophrenia. By contrast, levels of [<sup>3</sup>H]MK-801, [<sup>3</sup>H]AMPA, [<sup>3</sup>H]aspartate, or [<sup>3</sup>H]CGP39653 binding did not differ between the diagnostic cohorts. Levels of mRNA for the GluR5 subunit were decreased overall ( $p < 0.05$ ), with no changes in levels of mRNA for GluR6, GluR7, KA1, or KA2 in tissue from subjects with schizophrenia. These data indicate that the decreased number of kainate receptors in the dorsolateral prefrontal cortex in schizophrenia may result, in part, from reduced expression of the GluR5 receptor subunits.

Neuropsychopharmacology (2005) 30, 1521–1531 advance online publication, 11 May 2005; doi:10.1038/sj.npp.1300758

**Keywords:** receptors; glutamate; mental disorders; human; *in situ* hybridization; autoradiography

## INTRODUCTION

Glutamate, the most abundant and best characterized of the excitatory amino acids in the central nervous system (CNS), is thought to influence the activity of most neurons (Fagg and Foster, 1983). Glutamate acts through a number of receptors that affect both fast and slow neurotransmission and are accordingly divided into ionotropic and G-protein-coupled metabotropic receptors (Hollmann and Heinemann, 1994). The family of pharmacologically distinct ionotropic receptors is made up of the *N*-methyl-D-aspartate (NMDA) receptor, the amino-3-hydroxy-5-methyl-4-isoxazole (AMPA) receptor, and the kainate receptor. Each ionotropic receptor is a multimeric aggregation of different protein subunits that combine to form a ligand-gated ion channel. For the NMDA receptor, NMDAR1 is the obligate subunit and combines with NMDAR2A–2D subunits to

form a gated channel. AMPA receptors are formed from four different subunits, termed GluR1–4, all of which can exist in two different forms, while the five different kainate subunits (GluR5–7 and KA1–2) can undergo alternative splicing and editing (Nakanishi, 1992).

The cornerstone of the glutamatergic hypothesis of schizophrenia is the observation that phencyclidine (PCP, angel dust) can induce psychoses similar to that associated with schizophrenia (Luisada, 1978) and binds to the NMDA receptor (Loo *et al*, 1987). Adding weight to the argument that the NMDA receptor is involved in the pathology of schizophrenia is the neuropsychopharmacological observation that ketamine, which is a noncompetitive NMDA antagonist (White *et al*, 1982), increases the severity of the positive symptoms of schizophrenia (Lahti *et al*, 1995). In addition, after receiving ketamine, some of the schizophrenic subjects who received the drug reported that ‘they felt as if they had stopped their antipsychotic medication’ (Tamminga, 1999). Thus, data from neuropsychopharmacological studies have focused attention on the NMDA receptor and its possible role in the pathology of schizophrenia.

Studies using post-mortem CNS from subjects with schizophrenia have attempted to understand changes in the NMDA receptor in schizophrenia, but have not yet fully

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Received 18 January 2005; revised 23 March 2005; accepted 24 March 2005

Online publication: 1 April 2005 at <http://www.acnp.org/citations/Npp040105050037/default.pdf>

defined the mechanisms by which changes in the NMDA receptor could induce symptoms associated with the disorder. However, such studies have revealed region-specific changes in NMDA and other ionotropic glutamate receptors at the level of protein and mRNA in brain tissue from subjects with the disorder. In a recent review of the available post-mortem data (Meador-Woodruff and Healy, 2000), it was highlighted that there are some consistencies regarding glutamate receptors in schizophrenia. These include decreased expression of the NMDAR1 subunit for the NMDA receptor in some cortical areas, decreases in both kainate and AMPA receptors in the hippocampal formation, and a decrease in kainate but not AMPA receptors in the cortex from subjects with the disorder. Overall, this review supported the hypothesis that abnormalities in cortical glutamatergic ionotropic receptors are a critical component of the changes in the glutamatergic system in subjects with schizophrenia.

Of particular relevance to the glutamatergic hypothesis of schizophrenia are recent reviews of genetic studies (Harrison and Weinberger, 2005; Owen *et al*, 2005), which highlight associations between the expression of polymorphisms in genes for proteins associated with synapses, particularly glutamatergic synapses, with increased susceptibility to schizophrenia. Three such proteins are (i) dysbindin 1, overexpression of which has been shown to increase glutamate release by neuronal cultures (Numakawa *et al*, 2004); (ii) neuregulin 1, heterozygote knockouts of which have lower NMDA receptors and showed abnormal prepulse inhibition (an animal model used to predict antipsychotic activity in compound development) and hyperactivity in novel environments (Stefansson *et al*, 2002); and (iii) G72, a protein that activates D-amino-acid oxidase (DAAO), one of the enzymes involved in metabolizing D-serine, known to be an agonist at one of the NMDA receptor modulatory sites (Chumakov *et al*, 2002). The convergence of the products of these three susceptibility genes on the glutamatergic system augments the neuropsychological and post-mortem work emphasizing a role for the system in the pathology of schizophrenia.

A growing body of data supports the position that the functioning of the glutamatergic system relies on a high degree of interactivity between ionotropic glutamate receptors. For example, presynaptic kainate (Perkinton and Sihra, 1999) and NMDA (Breukel *et al*, 1998) receptors modulate glutamate release, while both kainate and AMPA receptors act to depolarize the postsynaptic membrane, reducing the ion channel block at the NMDA receptor (Goff and Coyle, 2001). These data suggest that a comprehensive study of ionotropic glutamate receptors within the same cohorts of subjects is most likely to provide insight into how changes in these receptors might precipitate the onset of the symptoms of schizophrenia. Thus, to gain a more global view of changes in cortical ionotropic glutamate receptors in schizophrenia, we have measured the densities of NMDA ( $[^3\text{H}]\text{MK-801}$  binding (open ion channels) and  $[^3\text{H}]\text{D,L-(E)-2-amino-4-propyl-5-phosphono-3-pentanoic acid}$  ( $[^3\text{H}]\text{CGP39653}$ ) binding (glutamate binding site)), AMPA ( $[^3\text{H}]\text{AMPA}$  binding), and kainate ( $[^3\text{H}]\text{kainate}$  binding) receptors, as well as that of the high-affinity glutamate transporter ( $[^3\text{H}]\text{aspartate}$ ) in Brodmann's area (BA) 9 obtained from subjects with the disorder and subjects with

no history of psychiatric illness (controls). In order to determine whether the changes we saw in radioligand binding were associated with changes in transcript expression, we have also measured levels of mRNA for all of the kainate receptor subunits using *in situ* hybridization.

## MATERIALS AND METHODS

$[^3\text{H}]\text{CGP39653}$ ,  $[^3\text{H}]\text{MK-801}$ ,  $[^3\text{H}]\text{AMPA}$ ,  $[^3\text{H}]\text{kainate}$ , and  $[^3\text{H}]\text{aspartate}$  were obtained from New England Nuclear. L-Glutamic acid, quisqualic acid, spermidine, and potassium thiocyanate were obtained from Sigma-Aldrich Pty. Ltd, as were all other laboratory-grade reagents. Kodak BIOMAX MR film and D19 developer were obtained from Kodak, Rochester, NY. Hyperfilm  $[^3\text{H}]$ ,  $[^3\text{H}]\text{microscales}^{\text{TM}}$ , and  $[^{14}\text{C}]\text{microscales}^{\text{TM}}$  were obtained from Amersham Biosciences Pty., Ltd.

## Tissue Preparation

Consent for the study was obtained from the Ethics Committee of the Victorian Institute of Forensic Medicine and the North Western Mental Health Program Behavioural and Psychiatric Research and Ethics Committee. Post-mortem tissue used in this study was obtained at autopsy and held in the CNS repository at the Rebecca L Cooper Research Laboratories. Blocks of tissue, containing BA 9, were removed from the left cerebral cortex of 20 subjects with a preliminary diagnosis of schizophrenia and 20 subjects, matched for age and sex, with no history of psychiatric illness (controls). The diagnosis of schizophrenia was confirmed from case histories, according to DSM-IV criteria (American Psychiatric Association, 1994), by a senior psychologist and a psychiatrist using the Diagnostic Instrument for Brain Studies (Hill *et al*, 1999). The duration of illness (DOI—the interval between the first hospital admission and time of death) and the last recorded antipsychotic drug doses, expressed as chlorpromazine equivalents (Remington, 1999), were calculated (Table 1). The pH of the tissue was measured to assess the agonal status of the brain (Kingsbury *et al*, 1995). Where the death was witnessed, the post-mortem interval (PMI) was the time between death and autopsy. In cases where the death was not witnessed, the PMI was taken as being half of the interval between the individual last being seen alive and being found dead. Cases were accepted when less than 5 h elapsed between the subject last being seen alive and being found dead. All samples were stored frozen at  $-70^{\circ}\text{C}$  from the time of autopsy.

## In Situ Radioligand Binding

A total of 20 sections ( $20\text{ }\mu\text{m}$ ) were cut from the BA 9 blocks taken from the lateral surface of the frontal lobe, within the region bordered by the middle frontal gyrus superior and the inferior frontal sulcus. The sections were thaw mounted on gelatinized slides.

The binding densities of the radioligands  $[^3\text{H}]\text{MK-801}$ ,  $[^3\text{H}]\text{CGP39653}$ ,  $[^3\text{H}]\text{AMPA}$ ,  $[^3\text{H}]\text{kainate}$ , and  $[^3\text{H}]\text{D-aspartate}$  were essentially determined as previously described. Thus, for the determination of  $[^3\text{H}]\text{MK-801}$  binding, sections were preincubated in assay buffer (50 mM

**Table 1** Demographics of Subjects from whom Tissue was Used for the Study of Cortical Glutamate

Schizoph	Age	Sex	PMI	Brain pH	Antipsychotic (mg, schedule)	Chlor. Eq.
1	67.0	M	21.0	6.46	Fluphenazine (37.5, fortnight)	75.0
2	22.0	M	37.0	6.07	Trifluoperazine (15); flupenthixol (40, 4 weeks)	450.0
3	40.0	F	61.0	5.71	Fluphenazine (25, fortnight; 5)	550.0
4	53.0	M	37.0	5.98	Fluphenazine (75, fortnight); Chlorpromazine (800)	1700.0
5	81.0	F	25.0	6.31	Trifluoperazine (5)	100.0
6	35.0	M	47.0	6.27	Fluphenazine (50, 3 weeks)	400.0
7	63.0	M	73.0	6.07	Trifluoperazine (10); chlorpromazine (100)	300.0
8	32.0	M	17.0	6.05	Haloperidol (200, 4 weeks)	670.0
9	46.0	M	42.0	5.53	Haloperidol (50, 4 weeks)	160.0
10	22.0	M	37.0	6.17	Pimozide (4)	200.0
11	68.0	F	42.0	5.73	Trifluoperazine (20)	400.0
12	42.0	M	34.5	6.26	Flupenthixol (40, 3 weeks); chlorpromazine (500)	610.0
13	38.0	M	50.0	6.02	Clozapine (50)	100.0
14	53.0	M	43.0	6.23	Chlorpromazine (200)	200.0
15	71.0	M	48.0	6.45	Thioridazine (150)	150.0
16	35.0	F	39.0	6.38	Resperidone (3)	150.0
17	69.0	M	44.5	6.38	Trifluoperazine (5)	100.0
18	42.0	M	47.0	6.26	Fluphenazine (75, fortnight)	1000.0
19	47.0	M	41.5	6.52	Chlorpromazine (400); haloperidol (150, fortnight)	1400.0
20	61.0	M	37.5	6.46	Fluphenazine (62.5, fortnight)	745.5
Mean $\pm$ SEM	49.35 $\pm$ 3.8		41.20 $\pm$ 2.8	6.16 $\pm$ 0.06		473.0 $\pm$ 100.7
Controls	Age	Sex	PMI	Brain pH		
1	65.0	M	20.5	6.47		
2	22.0	M	51.0	6.58		
3	38.0	F	50.5	6.14		
4	53.0	M	44.5	6.56		
5	80.0	F	55.0	6.28		
6	35.0	M	27.0	6.40		
7	65.0	M	41.0	6.56		
8	35.0	M	55.0	6.52		
9	46.0	M	46.5	6.08		
10	22.0	M	62.0	6.39		
11	66.0	F	43.0	6.37		
12	43.0	M	45.0	6.25		
13	42.0	M	63.0	6.34		
14	48.0	M	52.0	5.99		
15	72.0	M	39.0	6.21		
16	33.0	F	42.0	6.41		
17	68.0	M	41.0	6.06		
18	38.0	M	46.0	6.42		
19	42.0	M	43.0	6.61		
20	67.0	M	32.0	6.14		
Mean $\pm$ SEM	49.00 $\pm$ 3.8		45.0 $\pm$ 2.4	6.34 $\pm$ 0.04		

Schizoph = schizophrenics, age is given in years, PMI = post-mortem interval (h), Chlor. Eq. = chlorpromazine equivalents (mg).

Tris-acetate (pH 7.4) containing 100  $\mu$ M glutamate, 50  $\mu$ M glycine, and 50  $\mu$ M spermidine) at room temperature (RT) for 30 min. The specific binding of [ $^3$ H]MK-801 (20 nM) was

defined as the difference in radiolabel binding in the absence (TB) and presence (NS) of MK-801 (100  $\mu$ M) following a 60 min incubation, in assay buffer, at RT

(Johnson *et al*, 1993). [ $^3\text{H}$ ]CGP39653 binding density was determined following a 45 min preincubation in assay buffer (50 mM Tris-HCl, pH 8) at RT. Sections were incubated in assay buffer with [ $^3\text{H}$ ]CGP39653 (20 nM) in the absence (TB) or presence (NS) of L-glutamic acid (1 mM) for 45 min at RT (Ibrahim *et al*, 2000a). Determination of [ $^3\text{H}$ ]AMPA binding followed a 30 min preincubation in assay buffer (50 mM Tris-HCl (pH 7.4) containing 2.5 mM  $\text{CaCl}_2$  and 0.1 M potassium thiocyanate) at RT. Nonspecific sections were incubated with [ $^3\text{H}$ ]AMPA (100 nM) in the presence of 100  $\mu\text{M}$  quisqualic acid, while those for total binding were incubated with [ $^3\text{H}$ ]AMPA (100 nM) only in assay buffer for 45 min at 4°C (Jansen *et al*, 1990). Sections for the determination of [ $^3\text{H}$ ]kainate were first preincubated in assay buffer (50 mM Tris-acetate, pH 7.4) for 30 min at RT. Then, the sections for total binding were incubated with [ $^3\text{H}$ ]kainate (40 nM) while those for nonspecific binding were incubated in the presence of [ $^3\text{H}$ ]kainate (40 nM) and L-glutamic acid (1 mM) in assay buffer for 60 min at 4°C (Greenamyre and Young, 1989). Binding of [ $^3\text{H}$ ]aspartate was determined following a 20 min preincubation in assay buffer (50 mM Tris-acetate with 300 mM NaCl, pH 7.4) at 4°C. Sections for the determination of total binding were then incubated with 40 nM [ $^3\text{H}$ ]D-aspartate, while those for the determination of nonspecific binding were incubated with 40 nM [ $^3\text{H}$ ]D-aspartate in the presence of 200  $\mu\text{M}$  D-aspartate in the assay buffer at 4°C for 20 min (Noga *et al*, 1997).

Following their incubations with the respective radioligands, all sections were washed twice in ice-cold assay buffer and then dipped in distilled water, to remove salts, before being dried in a stream of cool air. Sections used for the determination of [ $^3\text{H}$ ]MK-801, [ $^3\text{H}$ ]AMPA, [ $^3\text{H}$ ]kainate, and [ $^3\text{H}$ ]D-aspartate were all apposed to Hyperfilm-[ $^3\text{H}$ ]™ with [ $^3\text{H}$ ]microscales™ until an image suitable for analysis was obtained (AMPA, 14 days; kainate, 30 days; MK-801, 5 weeks; D-aspartate, 4 months). The film was then developed in Kodak D19 developer and fixed in Hypam fixative. The images were analyzed from film using the MCID M1 image analysis system. The sections used for the determination of [ $^3\text{H}$ ]CGP39653 were partially fixed overnight in paraformaldehyde fumes at RT, to prevent contamination of the unshielded tritium-sensitive phosphoimage plates, before being apposed to BAS-TR2025 phosphoimage plates with [ $^3\text{H}$ ]microscales™ until an image suitable for analysis was obtained (14 days). The phosphoimage plates were then scanned using a BAS5000 high-resolution phosphoimager and the images analyzed using AIS image analysis software.

For all autoradiographs, the density of the images was converted to dpm/mg estimated tissue equivalent (ETE) by comparison with the standard curve generated using the relative densities of differing levels of radioactivity present on discrete zones of the [ $^3\text{H}$ ]microscales™. Using the specific activities of the appropriate radioligand, the values were converted to fmol/mg ETE.

As discussed previously (Scarr *et al*, 2003), all binding studies were of the single-point saturation design, where all available receptor sites are occupied by radioligand because the binding of each radioligand was measured at a concentration at least three times that of the  $K_D$  of the ligand for the receptor of interest (Hoffman *et al*, 1997; Nishikawa *et al*, 1983; Piggott *et al*, 1992; Sills *et al*, 1991).

Hence, specific binding (SB) of each radioligand, calculated as total binding (TB, three sections) minus the binding of the radioligand in the presence of a nonradioactive drug that competes for the same binding site (nonspecific, NSB, two sections), that is,  $\text{SB} = \text{TB} - \text{NSB}$ , is a good estimate of the density of binding sites in the tissue studied (Rodbard, 1981).

### In Situ Hybridization

A total of 10 sections (14  $\mu\text{m}$ ) were cut from the same BA 9 blocks used for the *in situ* radioligand binding studies and thaw mounted on Superfrost slides.

Transcripts were detected using subclones as previously described (Healy and Meador-Woodruff, 1997; Ibrahim *et al*, 2000b; Ibrahim *et al*, 2000a; Meador-Woodruff *et al*, 1996, 2001b). Briefly, riboprobes were synthesized from linearized plasmids containing subclones corresponding to regions common to all splice and edited variants of each kainate receptor subunit transcript (summarized in Table 2). All subclones were prepared in either pBluescript SK(–) or pBluescript KS(–), ranged from 469 to 865 bases, had similar G/C ratios (<50%), encoded specific fragments of each sequence (Table 2), and were confirmed by nucleotide sequencing.

Riboprobes were synthesized using 100  $\mu\text{Ci}$  of dried [ $^{35}\text{S}$ ]-UTP, 2.0  $\mu\text{l}$  5 $\times$  transcription buffer, 1  $\mu\text{l}$  0.1 M DTT, 1  $\mu\text{l}$  each of 10 mM ATP, CTP, and GTP, 1  $\mu\text{g}$  of linearized plasmid DNA, 0.5  $\mu\text{l}$  RNA inhibitor, and 1.5  $\mu\text{l}$  of T3 or T7 RNA polymerase. After incubating for 2 h at 37°C, 1.0  $\mu\text{l}$  DNase (RNase-free) was added and incubated for 15 min at RT. The reaction mixture was then passed through Micro Bio-Spin P-30 Tris Chromatography Columns and the purified fractions eluted. DTT was added to each fraction at a final concentration of 0.01 M.

For each probe, two sections per subject were removed from –80°C storage, fixed in 4% (w/v) formaldehyde for 1 h at RT, and rinsed three times in 2 $\times$  SSC (standard saline citrate; 300 mM sodium chloride and 30 mM sodium citrate, pH 7.2). The sections were treated with 0.1 M triethanolamine (pH 8.0)/acetic anhydride, 400:1 (vol:vol), with stirring for 10 min at RT, rinsed in 2 $\times$  SSC for 5 min, and dehydrated through graded alcohols before being air dried.  $^{35}\text{S}$ -labelled probes were applied to the slides after diluting 3–5 $\times$  10<sup>6</sup> cpm in 400  $\mu\text{l}$  of hybridization buffer consisting of 50% deionized formamide, 3 $\times$  SSC, 1 $\times$  Denhardt's solution (0.02% polyvinyl pyrrolidone, 0.02% Ficoll, and 0.02% bovine serum albumin), 2% yeast tRNA

**Table 2** Characteristics of the Probes used for *In Situ* Hybridization Studies

Probe	Accession number	Length (bp)	Region of gene in probe
GluR5	NM_017241	765	177–942
GluR6	Z11548	484	99–583
GluR7	M83552	865	1–865
KA1	NM_012572	607	1–531
KA2	NM_017262	469	1–469

(10 mg/ml), 50 mM sodium phosphate (pH 7.4), and 10% dextran sulfate in sterile water. Slides were covered with glass coverslips and placed in humid chambers saturated with 50% formamide at 55°C overnight. After 16 h of hybridization, the cover slips were removed and the sections washed three times in  $2 \times$  SSC at RT, immersed in RNase A (200 mg/ml in 10 mM Tris and 0.5 M NaCl, pH 8.0) for 30 min at 37°C, and then washed through decreasing concentrations of SSC to a final wash of  $0.1 \times$  SSC at 60°C for 2 h. Finally, the sections were dehydrated in graded ethanol solutions, allowed to air dry, placed in X-ray cassettes with [ $^{14}$ C]microscale standards, and exposed to Kodak BIOMAX MR film (Kodak, Rochester, NY) for 7–14 days. For each probe, all subjects were run in the same experiment to eliminate interassay variability (Ibrahim *et al*, 2000b; Meador-Woodruff *et al*, 2001a, b).

Images were acquired by digitizing film images using a CCD camera with NIH Image software (version 1.61). Gray-scale values were obtained for isodense bands identified for each probe, corrected for tissue background, and then converted to optical density (OD). OD values were then converted to amount of radioactivity bound (in nCi/g), determined from calibrated [ $^{14}$ C]microscale standards (Amersham Biosciences, Piscataway, NJ) (Akbarian *et al*, 1996; Downs and Williams, 1984; Williams, 1982), which had been exposed alongside the slides for each probe. Using the number of uridine residues contained in each subclone and specific activity of [ $^{35}$ S]UTP, bound radioactivity was converted to concentration of mRNA per isodense band, expressed as femtomoles of mRNA per gram of tissue (fmol/g) (Clinton *et al*, 2003; McCullumsmith *et al*, 2003).

## Statistical Analysis

For both *in situ* radioligand binding and *in situ* hybridization data, the Kolmogorov–Smirnov test was used to determine whether the data were normally distributed. Comparisons of both radiolabelled binding densities and mRNA levels across diagnostic groups and layers of binding were carried out with a two-way ANOVA. Where there were differences between the diagnostic groups, two-tailed unpaired *t*-tests were used to identify differences within each layer of binding. Binding densities for [ $^3$ H]CGP39653 and [ $^3$ H]D-aspartate were homogenously distributed across all cortical layers; therefore, the data for these ligands were analyzed using two-tailed unpaired *t*-tests. Relationships between experimental parameters and potential confounding factors (PMI, age, pH, psychotropic drug equivalents, and DOI) were analyzed using Pearson's single product moment correlations calculated using an assumed straight line fit. All analyses were carried out using GraphPad Prism Version 4.0 for Windows (GraphPad Software Inc., San Diego, California, USA).

## RESULTS

### Demographics

There were no significant differences in age or PMI between the diagnostic cohorts. Brain pH was significantly lower in the schizophrenic group compared to that of the controls ( $6.17 \pm 0.06$  vs  $6.34 \pm 0.04$ ,  $p = 0.025$ ) (Table 1). However,

there were no significant correlations between the demographic factors for either diagnostic group and measurements of either radioligand binding density or subunit mRNA levels ( $r^2$  values range from 0.494 to 0.0004 for the control group and from 0.438 to 0.0027 for the schizophrenic group).

### *In Situ* Radioligand Binding

As the bindings of [ $^3$ H]CGP39653 and [ $^3$ H]D-aspartate were homogenously distributed across all cortical laminae (Figure 1a and b, respectively, insets show nonspecific binding), a single measure of radioligand binding was taken across the cortex. By contrast, [ $^3$ H]MK-801 and [ $^3$ H]AMPA bindings were distributed in two distinct bands (Figure 1c and d, respectively, insets show nonspecific binding), which corresponded to cortical laminae I–III and IV–VI (MK-801) and cortical laminae I–II and III–VI (AMPA), respectively. [ $^3$ H]Kainate binding was different in that it was distributed in three distinct bands (Figure 1e, inset shows nonspecific binding); these corresponded to cortical laminae I–II, III–IV, and V–VI.

### NMDA Receptors

There was no significant variance in levels of [ $^3$ H]MK-801 binding with diagnosis ( $F = 0.44$ , d.f. = 1,76,  $p = 0.507$ ; Table 3), but there was significant variance in radioligand binding between layers ( $F = 13.24$ , d.f. = 1,76,  $p < 0.001$ ) with no significant interaction between these variables ( $F = 0.18$ , d.f. = 1,76,  $p = 0.675$ ). Two-tailed unpaired *t*-tests showed that the variation in [ $^3$ H]MK 801 binding was due to a lower level of binding in cortical laminae IV–VI compared to that in laminae I–III in control subjects ( $t = 3.362$ , d.f. = 38,  $p < 0.005$ ), with a strong trend to such a difference in the schizophrenic subjects ( $t = 2.018$ , d.f. = 38,  $p = 0.051$ ).

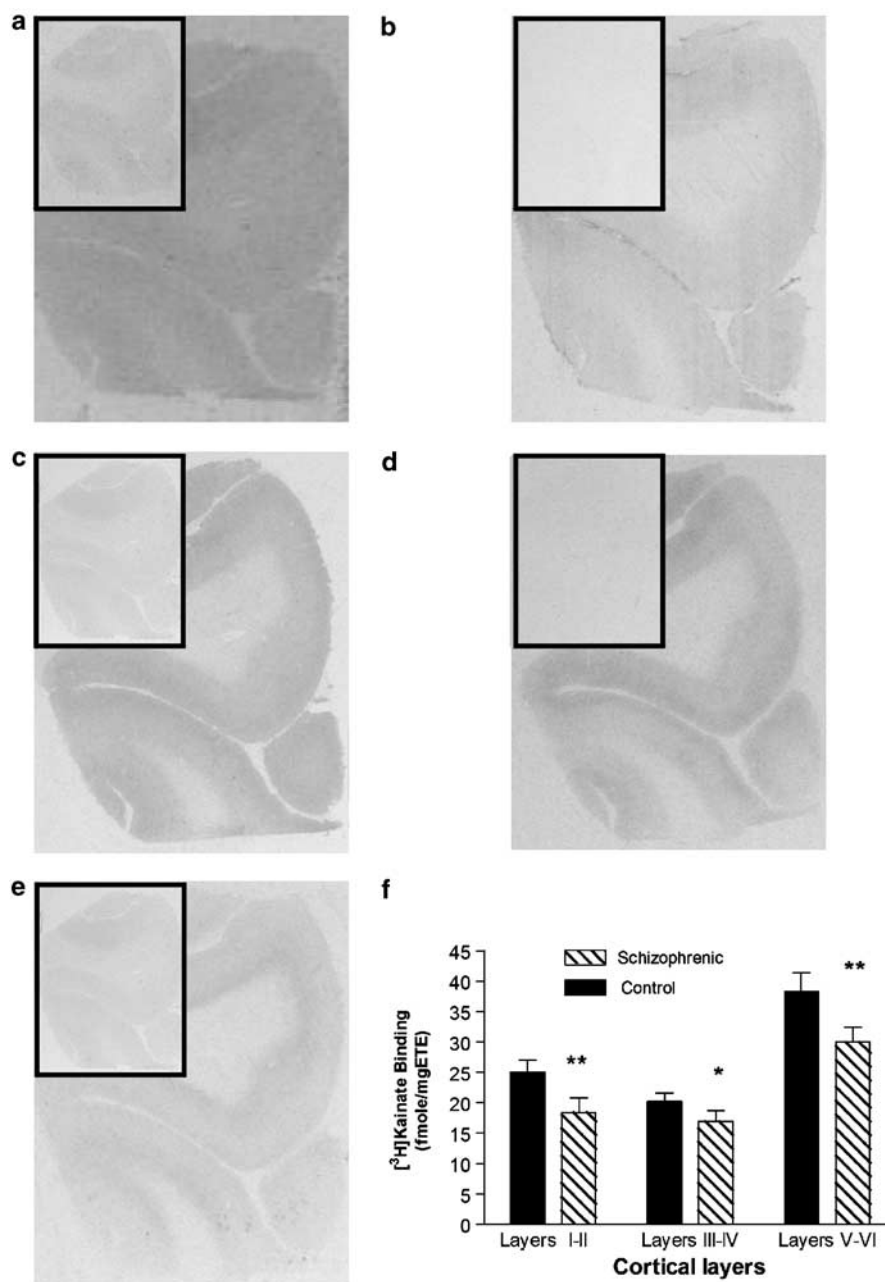
Analysis of [ $^3$ H]CGP39653 binding with two-tailed Student's unpaired *t*-test revealed no significant difference between tissue from subjects with schizophrenia and controls ( $t = 0.08$ , d.f. = 37,  $p > 0.05$ ; Table 3).

### AMPA Receptors

There was significant variance in [ $^3$ H]AMPA binding between layers ( $F = 18.01$ , d.f. = 1,74,  $p < 0.001$ ) but not between diagnosis ( $F = 1.70$ , d.f. = 1,74,  $p = 0.196$ ; Table 3), and there was no interaction between diagnosis and layer ( $F = 0.14$ , d.f. = 1,74,  $p = 0.713$ ). Further analysis between layers within diagnosis showed that the variation in radioligand binding was because of lower binding in laminae III–VI than in laminae I–II in both control ( $t = 3.746$ , d.f. = 36,  $p < 0.001$ ) and schizophrenic ( $t = 2.742$ , d.f. = 38,  $p < 0.01$ ) subjects.

### Kainate Receptors

There was significant variance in [ $^3$ H]kainate binding with diagnosis ( $F = 20.03$ , d.f. = 1,114,  $p < 0.001$ ; Figure 1f) and cortical layer ( $F = 37.92$ , d.f. = 2,74,  $p < 0.001$ ), but there was no interaction between diagnosis and layers ( $F = 0.48$ , d.f. = 2,114,  $p = 0.62$ ). Further analysis between diagnoses with two-tailed Student's unpaired *t*-test revealed significant



**Figure 1** Autoradiographs of BA 9 showing the distribution of (a)  $[^3\text{H}]\text{CGP39653}$ , (b)  $[^3\text{H}]\text{aspartate}$ , (c)  $[^3\text{H}]\text{MK-801}$ , (d)  $[^3\text{H}]\text{AMPA}$ , and (e)  $[^3\text{H}]\text{kainate}$  binding (insets show nonspecific binding). In addition, (f) is the graphical representation of  $[^3\text{H}]\text{kainate}$  binding (mean  $\pm$  SEM) in fmol/mg ETE in BA 9. \*\* $p \leq 0.01$ . The closed columns represent data from control subjects while the hatched columns represent data from schizophrenic subjects.

decreases between all layers of  $[^3\text{H}]\text{kainate}$  binding in tissue from subjects with schizophrenia compared to that from control subjects (layers I-II:  $t = 2.711$ , d.f. = 38,  $p = 0.01$ ; layers III-IV:  $t = 2.28$ , d.f. = 38,  $p < 0.05$ ; layers V-VI:  $t = 2.79$ , d.f. = 38,  $p < 0.01$ ). Analysis within diagnosis showed that  $[^3\text{H}]\text{kainate}$  binding in laminae I-II and III-IV was lower than in laminae V-VI for both diagnostic cohorts (controls:  $F = 21.35$ , d.f. = 2,57,  $p < 0.0001$ ; laminae I-II vs laminae V-VI  $p < 0.001$ ; laminae III-IV vs laminae V-VI  $p < 0.001$ ; Schizophrenics:  $F = 16.59$ , d.f. = 2,57,  $p < 0.0001$ ; laminae I-II vs laminae V-VI  $p < 0.001$ ; laminae III-IV vs laminae V-VI  $p < 0.001$ ).

### High-Affinity Glutamate Uptake Sites

Analysis of  $[^3\text{H}]\text{aspartate}$  binding with two-tailed Student's unpaired  $t$ -test revealed no significant difference between tissue from subjects with schizophrenia and that from controls ( $t = 0.75$ , d.f. = 38,  $p > 0.05$ ; Table 3).

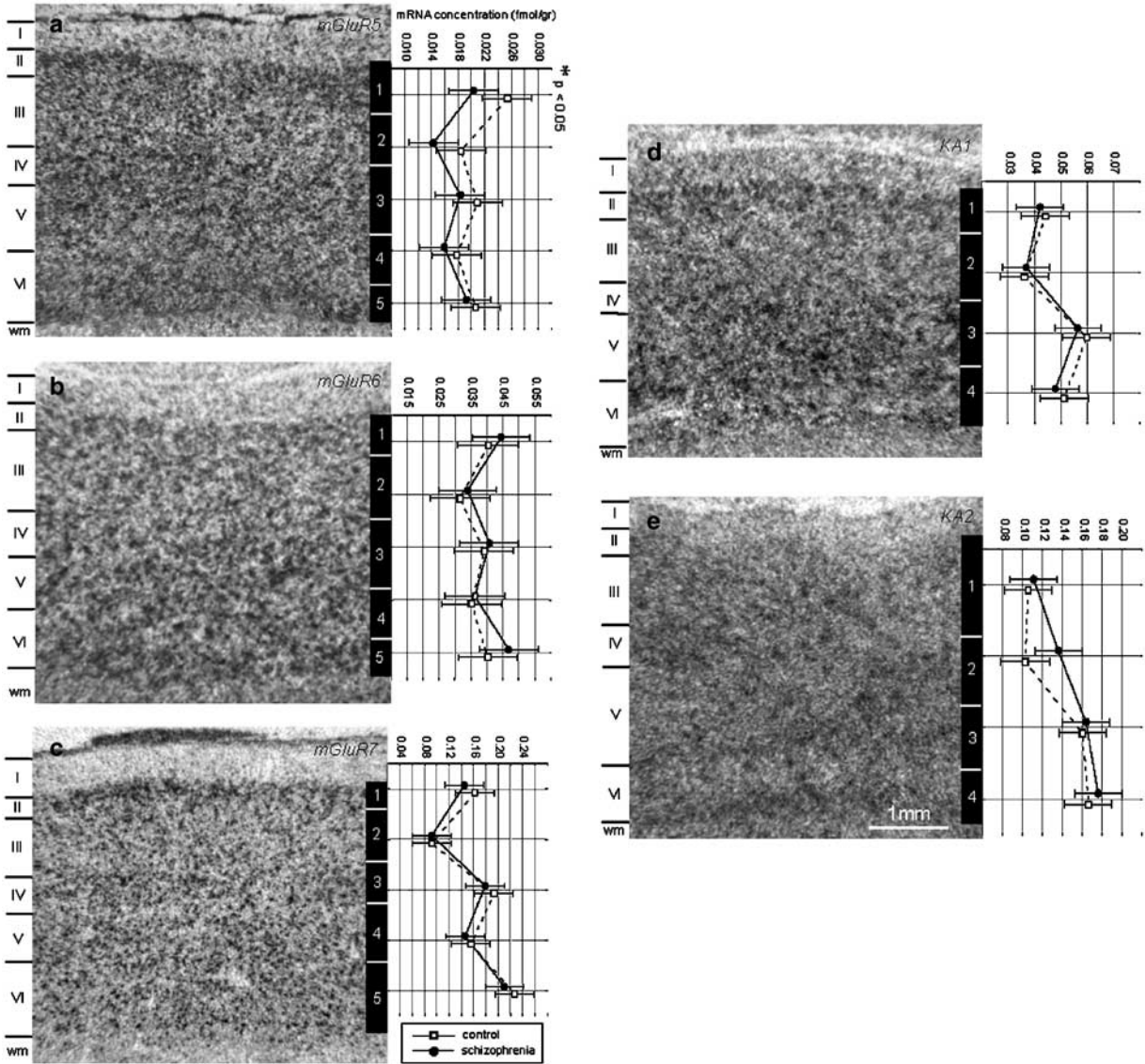
### In Situ Hybridization

The distribution for the low- (GluR5-7) and high-affinity kainate subunit (KA1 and 2) mRNA was visualized as five distinct isodense bands (Figure 2a-c) and four distinct

**Table 3** Summary of Radioligand Binding Data (fmol/mg ETE; mean  $\pm$  SEM) that was not Significantly Different between Diagnostic Cohorts

Radioligand	Control	Schizophrenic
[ <sup>3</sup> H]MK-801		
Layers I–III	99.56 $\pm$ 6.61	92.08 $\pm$ 8.65
Layers IV–VI	71.57 $\pm$ 5.07	69.88 $\pm$ 6.79
[ <sup>3</sup> H]CGP39653		
Layers I–VI	32.70 $\pm$ 2.51	33.05 $\pm$ 3.11
[ <sup>3</sup> H]AMPA		
Layers I–II	249.40 $\pm$ 11.01	272.90 $\pm$ 18.72
Layers III–VI	195.10 $\pm$ 9.43	208.20 $\pm$ 14.34
[ <sup>3</sup> H]aspartate		
Layers I–VI	35.95 $\pm$ 3.06	32.79 $\pm$ 2.87

isodense bands (Figure 2d and e), respectively. Comparison of the isodense bands with cortical laminae showed that the laminar distribution of mRNA was specific for each subunit. Isodense bands for GluR5 mRNA (Figure 2a) overlay cortical lamina II and the first third of lamina III (band 1), the remainder of lamina III (band 2), cortical lamina IV and the upper half of lamina V (band 3), the lower half of lamina V and the top quarter of lamina VI (band 4), and the deeper aspect of cortical lamina VI (band 5). Isodense bands for GluR6 mRNA (Figure 2b) overlay cortical lamina II and the top eighth of cortical lamina III (band 1), the remainder of cortical lamina III (band 2), lamina IV and the upper half of lamina V (band 3), the bottom half of lamina V and the upper half of lamina VI (band 4), and the lower half of lamina VI (band 5). For GluR7 mRNA (Figure 2c), isodense bands overlay cortical lamina II (band 1), III (band 2), IV (band 3), V (band 4),



**Figure 2** Images showing the distribution of mRNA for: (a) GluR5, (b) GluR6, (c) GluR7, (d) KA1, and (e) KA2 kainate receptor subunits across the dorsolateral prefrontal cortex. The cortical layers of each section are marked in their left margin. In their right margin, the graphs represent mRNA concentration in each of the isodense bands (black bar) in control (open squares) and schizophrenic (closed circles) subjects (mean  $\pm$  95% confidence limits) in femtomoles of mRNA per gram of tissue.

and VI (band 5). For the high-affinity subunits, the mRNA for KA1 isodense bands overlay cortical lamina II (band 1), III (band 2), IV and V (band 3), and VI (band 4). For KA2 mRNA, isodense bands overlay cortical laminae II and III (band 1) as well as lamina IV (band 2), V (band 3), and VI (band 3).

### High-Affinity Kainate Subunits

There was no significant variation in the levels of KA1 and KA2 subunit mRNA with diagnosis (KA1:  $F=0.36$ , d.f. = 1,148,  $p=0.55$ ; KA2:  $F=1.93$ , d.f. = 1,135,  $p=0.167$ ; Figure 2), but there was a variance between isodense bands for both subunits (KA1:  $F=8.14$ , d.f. = 3,148,  $p<0.0001$ ; KA2:  $F=17.94$ , d.f. = 3,135,  $p<0.0001$ ) without there being any interaction between these variables (KA1:  $F=0.09$ , d.f. = 3,148,  $p=0.97$ ; KA2:  $F=0.38$ , d.f. = 3,135,  $p=0.77$ ). Further analysis across isodense bands, within diagnoses, showed a significantly lower level of mRNA for KA1 in isodense band 2 compared to 3 (control:  $F=4.48$ , d.f. = 3,72,  $p<0.001$ , isodense band 2 vs isodense band 3  $p<0.01$ ; schizophrenic:  $F=3.65$ , d.f. = 3,76,  $p<0.05$ , isodense band 2 vs isodense band 3  $p<0.05$ ). For KA2 subunit mRNA, there were lower levels of mRNA in isodense bands 1 and 2 compared to isodense bands 3 and 4 (control:  $F=8.42$ , d.f. = 3,68,  $p<0.0001$ , isodense band 1 vs isodense band 3  $p<0.05$ ; isodense band 1 vs isodense band 4  $p<0.01$ ; isodense band 2 vs isodense band 3  $p<0.01$ ; isodense band 2 vs isodense band 4  $p<0.01$ ; schizophrenic:  $F=10.49$ , d.f. = 3,67,  $p<0.0001$ , isodense band 1 vs isodense band 3  $p<0.001$ ; isodense band 1 vs isodense band 4  $p<0.001$ ; isodense band 2 vs isodense band 3  $p<0.05$ ; isodense band 2 vs isodense band 4  $p<0.01$ ).

### Low-Affinity Kainate Subunits

Levels of GluR5 subunit mRNA varied with diagnosis ( $F=6.42$ , d.f. = 1,190,  $p<0.05$ ; Figure 2) and isodense bands ( $F=4.00$ , d.f. = 4,190,  $p<0.005$ ) without there being any significant interaction between these variables ( $F=0.34$ , d.f. = 4,190,  $p=0.85$ ). Further analysis between diagnoses with two-tailed Student's unpaired *t*-test failed to reveal significant differences in the levels of GluR5 subunit mRNA in any individual isodense band in subjects with schizophrenia compared to that in controls. Further analysis across isodense bands, within diagnoses, showed that in the control group, there was no variance associated with the different isodense bands ( $F=1.67$ , d.f. = 4,95,  $p=0.16$ ). However, in the schizophrenic group, there was variance associated with the isodense bands ( $F=3.87$ , d.f. = 4,95,  $p<0.01$ ); this was due to lower levels of GluR5 mRNA in isodense band 2 compared to isodense band 1 ( $p<0.01$ ).

Visual analysis of the data for GluR7 subunit mRNA suggested that some data points might be so variant that they would represent an outlier in comparison to the rest of the data set. This was confirmed with the Grubb's test, which showed that data from one control subject were outlier in four of the five isodense band measurements. There was no experimental reason to account for the increased variability in data from this subject and, therefore, to better assess the impact of the data on the

population analysis, variance was analyzed with and without the outlying data points. Thus, using the full data set, there was variance in the levels of GluR7 subunit mRNA with both diagnosis ( $F=4.94$ , d.f. = 1,190,  $p<0.05$ ) and isodense bands ( $F=12.7$ , d.f. = 4,190,  $p<0.0001$ ) with no interaction between the two factors ( $F=0.10$ , d.f. = 4,190,  $p=0.98$ ). Analysis of the proscribed data set showed no variance in the levels of GluR7 subunit mRNA with diagnosis ( $F=1.27$ , d.f. = 1,185,  $p=0.26$ ; Figure 2). There was variance associated with the isodense bands ( $F=17.46$ , d.f. = 4,185,  $p<0.0001$ ), but there was no interaction between the two factors ( $F=0.1$ , d.f. = 4,185,  $p=0.98$ ). The fact that data from a single subject could so profoundly affect statistical analyses added weight to the outcome from Grubb's analysis, which suggested that the data from one control subject were aberrant; therefore, further analysis was completed on a proscribed data set, which excluded the data from this particular subject.

Using the proscribed data, analysis between isodense bands, within diagnosis, showed that in both groups (control:  $F=8.18$ , d.f. = 4,90,  $p<0.0001$ ; schizophrenic:  $F=9.49$ , d.f. = 4,95,  $p<0.001$ ) this variance was associated with lower levels of GluR7 mRNA in isodense band 2 compared to isodense bands 3 (control  $p<0.01$ ; schizophrenic  $p<0.001$ ) and 5 ( $p<0.001$  for both groups), as well as lower levels in isodense band 4 compared to those in band 5 ( $p<0.05$  for both cohorts). In addition, in the control group, there is a higher level of GluR7 mRNA in isodense band 1 compared to isodense band 2 ( $p<0.05$ ), while in the schizophrenic cohort, there is a lower level of GluR7 mRNA in isodense band 1 than in isodense band 5 ( $p<0.05$ ).

There was no variance in levels of GluR6 subunit mRNA associated with diagnosis ( $F=1.53$ , d.f. = 1,185,  $p=0.22$ ; Figure 2) or isodense bands ( $F=1.75$ , d.f. = 4,185,  $p=0.14$ ) and no interaction between the variables ( $F=0.18$ , d.f. = 4,185,  $p=0.95$ ).

## DISCUSSION

This study has shown a decrease in the density of [ $^3\text{H}$ ]kainate binding in all cortical layers of BA 9 from subjects with schizophrenia. By contrast, there was no change in the levels of binding by the other four radioligands to other glutamatergic markers, including the NMDA and AMPA receptors, in tissue from the same cohorts of subjects. Under the conditions used in this study, [ $^3\text{H}$ ]kainate would bind to kainate receptors (Greenamyre and Young, 1989). Thus, our data are consistent with a decreased density of the kainate receptor in all cortical laminae from subjects with schizophrenia. *In situ* hybridization, using riboprobes directed against mRNAs for all of the kainate receptor subunits, showed a significant omnibus decrease in the levels of mRNA for the low-affinity kainate receptor subunit GluR5. Levels of mRNA for the other subunits were not different between diagnostic cohorts.

Previous studies have shown that changes in the glutamate receptors in the CNS from subjects with schizophrenia show regional specificity. This is demonstrated by studies that have investigated multiple cortical regions and shown an increase in the NMDA receptor in BA

22 but not BA 10 (Nudmamud and Reynolds, 2001) from subjects with the disorder. Similarly, decreases in kainate receptors in the prefrontal but not the occipital cortex (Meador-Woodruff *et al*, 2001a) have been reported. Thus, to avoid introducing cortical regional variation in changes in glutamate receptors as a confounding variable in discussion of our study in BA 9, our findings will be compared to other studies in either this CNS region or in the dorsolateral prefrontal cortex (DLPFC; BA 9 and 46) from subjects with schizophrenia.

Our finding of decreased cortical kainate receptors in BA 9 partially replicates another study, which showed a decrease in kainate binding in the prefrontal cortex (BA 9, 11, 32, and 46) in schizophrenia (Meador-Woodruff *et al*, 2001a). However, this earlier study found decreases in KA2 mRNA and increased GluR7 mRNA in tissue from the same subjects, which contrasts with our finding of decreased GluR5 mRNA. Other studies have reported an increase (Nishikawa *et al*, 1983; Toru *et al*, 1988) or no change (Dean *et al*, 2001; Noga *et al*, 2001) in the levels of kainate receptors in the DLPFC from subjects with schizophrenia.

There are numerous possible explanations for the variation in results on the study of kainate, and other glutamatergic, receptors in the cortex from subjects with schizophrenia, for example, smaller cohorts (eg  $n = 8$  in the previous study from one of our laboratories), the use of different levels of diagnostic criteria (eg schizophrenia *vs* catatonic, disorganized, paranoid, residual, and undifferentiated forms of the disorder; American Psychiatric Association 2000), differences in the genetic homogeneity of the diagnostic cohorts (eg Nishikawa *et al*, 1983; Toru *et al*, 1988, mainly Japanese), and different tissue collection parameters (eg average age of subjects; Meador-Woodruff *et al*, 2001a). It is clearly difficult to ascertain the impact of these variables. The present study was carried out on a substantial, well-defined cohort of subjects, and the radioligand binding and measurement of mRNA for the kainate subunits were carried out separately, with researchers blind to the coding sequence used for the samples. Therefore, considering our data, it could be argued that decreases in the levels of kainate receptors are present in BA 9 from subjects with schizophrenia and that these decreases could be due to decreased expression of mRNA for the GluR5 subunit.

Our finding that [ $^3\text{H}$ ]AMPA binding to the AMPA receptors is not changed in BA 9 from subjects with schizophrenia is in agreement with a number of previous studies in that region (Dean *et al*, 2001; Toru *et al*, 1988), the prefrontal cortex (BA 9, 11, 32, and 46) (Healy *et al*, 1998), and frontal cortex (region not specified) (Freed *et al*, 1993), although one study has reported an increase in the receptor in BA 46 with schizophrenia (Noga *et al*, 2001). Our study is novel in that cortical NMDA receptor density was measured using two radioligands, one of which binds within the ion channel ([ $^3\text{H}$ ]MK-801) (Wong *et al*, 1986) of the receptor while the other binds to the glutamate binding site on the receptor ([ $^3\text{H}$ ]CGP39653) (Sills *et al*, 1991). The binding of neither radioligand was altered in schizophrenia, which is in agreement with a number of earlier studies (Dean *et al*, 1999, 2001; Kornhuber *et al*, 1989; Noga *et al*, 2001) (region not specified in the Kornhuber study). Hence, overall current data seem to suggest that changes in NMDA

and AMPA receptors are not readily apparent in the DLPFC from subjects with schizophrenia.

Our finding of no change in [ $^3\text{H}$ ]aspartate binding in the DLPFC is in agreement with the only other study to be carried out in this area (BA 46) (Noga *et al*, 2001). These data would suggest that there is no change in the levels of high-affinity glutamate uptake sites in the DLPFC with schizophrenia.

Post-mortem studies in schizophrenia invariably have the confounding factor of the potential effects of pharmacotherapy prior to death. In this study, it was significant that there was no correlation between the levels of [ $^3\text{H}$ ]kainate binding or GluR5 mRNA and final recorded dose of antipsychotic drug. This suggests that there is no clear relationship between antipsychotic drugs received and changes in the kainate receptor in schizophrenia. In addition, another post-mortem study reported that mRNAs for NMDAR1, GluR1, GluR7, and KA1 subunits were lower in subjects with schizophrenia who were 'neuroleptic free' at death compared to those of controls and subjects treated with antipsychotics until death (Sokolov, 1998). These data infer that levels of mRNA for NMDAR1, GluR1, GluR7, and KA1 subunits can be increased because of antipsychotic drug treatment in humans. This hypothesis is partly supported by the report that clozapine, but not haloperidol, increases rat cortical mRNA for GluR7 (Healy and Meador-Woodruff, 1997), but this animal study suggests that changes in at least GluR7 mRNA may be drug class dependent. This is significant because in this study all schizophrenic subjects received typical antipsychotic drugs prior to death and it has been shown that treatment with such drugs either does not change (Spurney *et al*, 1999) or increases (Schmitt *et al*, 2003) levels of [ $^3\text{H}$ ]kainate binding in the rat cortex. Thus, together, these data would argue that our findings of decreased [ $^3\text{H}$ ]kainate binding and GluR5 subunit mRNA in tissue from subjects with schizophrenia are not simply a drug effect.

The impact of changes in kainate receptors on CNS function could be profound given that these receptors are both pre- (Vignes *et al*, 1998) and postsynaptic (Frerking *et al*, 1998), as well as being present on glial cells (Patneau *et al*, 1994). Furthermore, it is now accepted that the low-affinity kainate subunits (GluR5–7) can form functional receptors that are both homomeric and heteromeric, but that the high-affinity subunits only form heteromeric receptors (Hollmann and Heinemann, 1994). Our data suggest that the change in [ $^3\text{H}$ ]kainate binding may be associated with a change in expression of the GluR5 subunit, which could cause changes in both homomeric and heteromeric forms of the receptor. Significantly, in the rat hippocampus, it has been shown that receptors incorporating the GluR5 subunit are involved in the regulation of glutamatergic transmission (Vignes *et al*, 1998), which results in a decrease in the ability of receptor-specific agonists to activate either AMPA or NMDA receptors, an effect that can be blocked by a GluR5-preferring antagonist. In addition, it has also been shown that activating receptors incorporating the GluR5 subunit results in a reduction of  $\gamma$ -aminobutyric acid (GABA) inhibitory synaptic transmission (Clarke *et al*, 1997), an effect that is again reversed by the administration of a GluR5-preferring antagonist. Given that kainate can still

block hippocampal GABAergic transmission in GluR5 knockout mice, it would appear that the GluR5 is not the only subunit involved in kainite-mediated GABA interactions (Mulle *et al*, 2000), suggesting that heteromeric rather than homomeric receptors are required for this process. Projecting these data to the human cortex, the decrease in kainate receptors in schizophrenia containing the GluR5 subunit would likely affect the 'fine-tuning' of both inhibitory and excitatory synaptic transmission in the CNS. Furthermore, the ability of kainate receptors to modulate the activity of AMPA and NMDA receptors, without necessarily affecting their densities, implies that profound changes in glutamatergic transmission could still occur, despite there being no changes in the levels of radioligand binding to either AMPA or NMDA receptors in this cohort.

Although this study found that only one component of the cortical glutamatergic system was changed in schizophrenia, the fact that the kainate receptor, and in particular the GluR5-containing kainate receptor, has been implicated in modulating the release of both glutamate and GABA suggests that changes in this receptor could have a pervasive impact on the functioning of the cortical system. Decreasing the inhibitory input of the GABAergic system and the excitatory effect of the glutamatergic system would potentially not only destabilize the balance of information entering and leaving the cortex, via the predominantly glutamatergic ascending and descending pathways, but also the processing of this information within the cortex by changing the relative influence of GABAergic interneurons and cortical glutamatergic neurons.

## ACKNOWLEDGEMENTS

We thank Geoff Pavey for both technical assistance and as curator of the neural tissue repository, and Chris Hill, Ken Opeskin, David Copolov, and Nick Keks for their contribution to the tissue collection. Jaclyn McKenzie and Robyn Bradbury provided technical assistance. ES is a Ronald Phillip Griffith Research Fellow; this work was supported in part by a Stanley Research Centre Grant (03-RC-002).

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