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mRNA Expression of AMPA Receptors and AMPA Receptor Binding Proteins in the Cerebral Cortex of Elderly Schizophrenics

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L-α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors (AMPARs) mediate the majority of the fast excitatory transmission in the CNS. To determine whether gene expression of AMPARs and/or AMPAR binding proteins, which control response/sensitivity of AMPAR-bearing neurons to glutamate, are altered in schizophrenia, mRNA expression and abundance of AMPAR subunits (GluR1–4) and several AMPAR binding proteins (SAP97, PICK1, GRIP, ABP) were measured in the dorsolateral prefrontal cortex (DLPFC) and the occipital cortex of elderly schizophrenia patients ($n = 36$) and matched normal controls ($n = 26$) by quantitative real-time PCR. The mRNA expression of GluR1, GluR4, and GRIP in the DLPFC and expression of the GluR4, GRIP, and ABP in the occipital cortex were significantly elevated in schizophrenics. GluR1 and ABP mRNA expression in the occipital cortex and GluR2, GluR3, SAP97, and PICK1 expression in either cortical area were not significantly altered. The data also demonstrated significant differences in the abundances of mRNAs encoding GluR1–4 subunits (GluR2 > GluR3 > GluR1 > GluR4) and of AMPAR binding proteins (SAP97 > PICK1 > GRIP > ABP) in both diagnostic groups. GluR2 (58–64%) and GluR3 (24–29%) were the major components of the AMPAR mRNA in both cortical areas, implying that the major AMPAR complexes in the human cortex are probably those containing GluR2 and GluR3 subunits. Small but significant differences in the amounts of GluR2, GluR3, and GRIP mRNAs were detected between the two cortical areas: more GluR3 and GRIP but less GluR2 were detected in the DLPFC than in the occipital cortex.
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Key words: AMPA receptors; AMPA receptor binding proteins; mRNA expression; real-time PCR; schizophrenia; dorsolateral prefrontal cortex; occipital cortex

Glutamate is the primary excitatory neurotransmitter in the human brain. Excitatory synaptic activity conveyed by glutamate is mediated by three pharmacologically defined subtypes of ionotropic glutamate receptors: N-methyl-D-aspartate (NMDA), L-α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), and kainate. Studies conducted during the past decade have strongly implicated abnormalities in the glutamatergic system of the cerebral cortex and hippocampus in schizophrenia (Meador-Woodruff and Healy, 2000; Goff and Coyle, 2001; Harrison, 2004). Evidence for these abnormalities first emerged from pharmacological studies showing that antagonists of the NMDA subtype of glutamate receptors such as phencyclidine and ketamine can induce some of the psychotic signs and symptoms of schizophrenia in normal controls and can exacerbate symptoms in schizophrenics (Javitt and Zukin, 1991; Krystal et al., 1994, 1998, 1999; Halberstadt, 1995; Lahti et al., 1995a,b; Anand et al., 2000). These studies were instrumental in the development of a hypothesis that posits a hypofunctional postsynaptic glutamatergic system in schizophrenia (Carlsson et al., 2001). The mechanism of neuropsychiatric effects of NMDA antagonists, however, is not at all clear. An increase in dopamine release, effect on the γ-aminobutyric acid (GABA) system, and modulation of the serotonergic system have been suggested as possible mechanisms (Olney and Farber, 1995). The effects of NMDA antagonists may, in fact, be mediated by increased glutamate release (Moghaddam et al., 1997; Farber et al., 1998); phencyclidine and ketamine have been suggested to increase glutamatergic neurotransmission via non-NMDA receptors (e.g., AMPA and kainate; Moghaddam and Adams, 1998; Anand et al., 2000). These studies suggest that, contrary to the hypoglutamatergic theory of schizophrenia, the symptoms induced by NMDA antagonists could be a result of increased glutamate release and/or increased activation of non-NMDA postsynaptic glutamate receptors. Support for this possibility can be gleaned from studies that document increased ligand binding to AMPA receptors (AMPARs) in cortices of schizophrenics (Noga et al., 2001; Zavitsanou et al., 2002).

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AMPARs mediate the majority of the fast excitatory transmission in the CNS of vertebrates. These receptors are concentrated at postsynaptic densities (PSD) of excitatory synapses, although a large pool of AMPARs is also present in the cytoplasm of neuronal somata and dendrites (Petralia, 1997). AMPARs are tetrameric heterooligomers composed of combinations of glutamate receptor (GluR) subunits 1–4 (Wisden and Seeburg, 1993; Hollmann and Heinemann, 1994; Rosenmund et al., 1998). AMPAR trafficking between cytoplasmic compartments and cell membranes plays a prominent role in regulating synaptic efficacy. The number and type of AMPARs present at the PSD can be rapidly altered by synaptic activity through endocytosis and exocytosis of the receptors associated with the plasma membrane or intracellular membranes (Barry and Ziff, 2002; Malinow and Malenka, 2002).

AMPAR subunit C-terminal domains contribute to trafficking through interaction with cytosolic proteins that engage the trafficking machinery or anchor the receptor to different membranes. The GluR1 subunit C-terminus binds to SAP97 (for synapse-associated protein 97 kDa; Leonard et al., 1998), which belongs to the SAP90/PSD95 (for postsynaptic density protein 95 kDa) subfamily of membrane-associated guanylate kinase homologs (MAGUKs). The association of GluR1 and SAP97 appears to occur initially during the early stages of the biosynthesis of GluR1; an interaction between these two synaptic proteins has been detected, whereas GluR1 subunits are still in the endoplasmic reticulum and cis-Golgi network (Sans et al., 2001). SAP97 and GluR1 complexes appear to dissociate as the two proteins reach the synaptic plasma membrane, suggesting that SAP97 may play role in the synaptic trafficking of GluR1 (Sans et al., 2001; Rumbaugh et al., 2003).

The cytoplasmic tails of GluR2 and GluR3 are substantially homologous, and both bind the closely related multi-PDZ (PSD95/DLG/ZO-1) domain factors GRIP (for glutamate receptor interacting protein; Dong et al., 1997) and ABP (for AMPA receptor binding protein; Srivastava et al., 1998). In rodent brain, both GRIP and ABP are expressed in N-terminal leader palmitoylated and nonpalmitoylated isoforms (Yamazaki et al., 2001; DeSouza et al., 2002). These GRIP and ABP isoforms associate with the plasma membrane and spine heads and with intracellular membranes, respectively, and bind/anchor GluR2-containing AMPARs at these locations. The GluR2/3 cytoplasmic tails also bind PICK1 (for protein interacting with C kinase 1), a single PDZ domain protein that clusters AMPARs (Xia et al., 1999).

We have recently demonstrated an increased mRNA expression of two NMDA receptor subunits (NR1 and NR2A) and PSD95, which forms a postsynaptic scaffold for the NMDA receptor, in the dorsolateral prefrontal cortex (DLPFC) and the occipital cortex of elderly, chronically ill schizophrenics relative to normal controls (Dracheva et al., 2001). Given the pivotal role of AMPARs in glutamatergic neurotransmission and their potential involvement in mediating the effects of psychotomimetics, such as ketamine, we sought to determine whether the expression of genes coding for the GluR1–4 subunits of the AMPAR and/or for their binding proteins SAP97, ABP, GRIP, and PICK1 are increased in the cortices of schizophrenics.

TABLE I. Demographic Characteristics of the Study Cohort

	N	Sex (M:F)	PMI (hr)	Age at death (years)	pH
Normal controls	26	11:15	8.6 ± 1.2	80.9 ± 2.2	6.38 ± 0.05
Schizophrenics	36	27:9	14.0 ± 1.5	75.6 ± 1.9	6.41 ± 0.05

MATERIALS AND METHODS

Human Post-Mortem Tissue

Frozen post-mortem brain tissue from the DLPFC (Brodmann area 46) and visual cortex (Brodmann area 17) of subjects diagnosed with schizophrenia (N = 36) by DSM-IV criteria and normal elderly controls (N = 26) were obtained from the Mount Sinai/Bronx Veterans Administration (VA) Medical Center Department of Psychiatry Brain Bank. The mean age, post-mortem interval, tissue pH, and sex distributions of the cohorts used in this study are shown in Table I. All schizophrenic subjects had been chronically hospitalized at Pilgrim Psychiatric Center (New York) or associated nursing homes for many years. The Written Informed Consents were obtained from the next of kin. All assessment and post-mortem procedures were approved by the Institutional Review Boards of Pilgrim Psychiatric Center, Mount Sinai School of Medicine, and the Bronx VA Medical Center. All patients had thorough neuropathologic characterization to rule out discernible neuropathologies such as Alzheimer's disease and multiinfarct dementia (Purohit et al., 1998). Normal controls had no history of any psychiatric or neurologic disorders and no discernible neuropathologic lesions. All subjects, normal controls and schizophrenics, died of natural cause. Among the schizophrenia subjects, nine had been off neuroleptic medications for at least 6 weeks prior to death (range off neuroleptics = 0–246 weeks). The dissections of tissues were performed as described by Dracheva et al. (2001).

Quantitation of mRNA Expression

RNA isolation and RT reaction. Total RNA extraction and reverse transcriptase (RT) reactions were performed as described previously (Dracheva et al., 2001, 2003, 2004). Template RNA quality, including degradation and DNA contamination, was controlled as described previously (Dracheva et al., 2001, 2003, 2004). The resulting cDNA preparations were diluted 25 times in water; 5 μl of the diluted cDNA were amplified in 25 μl of PCR.

Primer, molecular beacon, and TaqMan design. Real-time PCR was used to determine the relative mRNA expression of AMPAR subunits (GluR1–4) and AMPAR binding proteins (SAP97, ABP, GRIP, and PICK1; Tyagi and Kramer, 1996; Bonnet et al., 1999; Marras et al., 1999; Vet et al., 1999). The human ABP has not yet been identified; how-

TABLE II. Primers, Molecular Beacons, and TaqMan Probes Used for the Detection of mRNA Expression*

Target	Primers	Accession No.	Primer position	Product size (bp)
β-Actin MB and TaqMan	F-TCACCCACACTGTGCCCATCTACGA	NM_001101	549–573	295
	R-CAGCGGAACCGCTCATTGCCAATGG		843–819	
GluR1	F-GGAAGGACGGGACCAGACAA	NM_000827	1856–1875	133
	R-AACGATGCGACCAGACAGGG		1988–1969	
GluR2	F-AGTGCGGAGCCCTCTGTGTT	NM_000826	2249–2268	137
	R-ATGGTGTCGCAAGGCTTCCT		2385–2366	
GluR3	F-TGCCAATCTCGCTGCTTTCC	NM_000828	2223–2242	107
	R-CGGAGTCCAGGGTCCCATAT		2329–2310	
GluR4	F-CAGAAGAGCCAGAGGACGGA	NM_000829	1724–1743	136
	R-CCTGAGAGGGATCTGGGTGA		1859–1840	
SAP97	F-GAGAGCTCAGAAAAGGAGATCGTATT	NM_004087	1699–1724	79
	R-GCTGCTGCCTGCTCATGAC		1777–1758	
ABP	F-CTGACACGGTGGCTAATGCTT	AB051506	731–751	60
	R-ACCCTGGCGTCTTGACTATTTC		790–769	
GRIP	F-GGCTTACTGGAGAAAGGAGTGTATG	XM_047362 (PDZ7)	605–629	101
	R-TGATTCACCTGTAAGAGCCTGTCA		705–682	
PICK1	F-CCTGCCTCTATATCGTCCAGGTA	NM_012407	361–383	71
	R-ATCGCCAGCTGCCACTGT		431–414	

	Probes
β-Actin MB	FAM-CCGGTC AGCCGTGGCCATCTCTTGCTCGAAG GACCGG-DABCYL
GAPDH MB	FAM-CGCAGC CGCCAGTAGAGGCAGGGATGAT GCTGCG-DABCYL
GluR1 MB	TET-CTGCGC CCTTGCTGCATGAAGGCTCCCA GCGCAG-DABCYL
GluR2 MB	TET-CTGCGC GTGGCTAGAGTGCGGAAGTCCA GCGCAG-DABCYL
GluR3 MB	TET-CGCTCG AGCTAAGTCTTCAGCACTCTCTATGGG CGAGCG-DABCYL
GluR4 MB	TET-CGCAGC GACCCAGCGACCAGCCTCCCA GCTGCG-DABCYL
β-actin TaqMan	VIC-ATGCCCTCCCCCATGCCATCCTGCGT-TAMRA
SAP97 TaqMan	FAM-CAGCTCTGAGGTCAACA-MGB
ABP TaqMan	FAM-CACCATCAAGGGTCC-MGB
GRIP TaqMan	FAM-TGGGCCAGGAGATC-MGB
PICK1 TaqMan	TET-CCGTCCAAGGCTGCTG-MGB

*FAM, fluorescein; TET, tetrachlorofluorescein; VIC, Applied Biosystems proprietary dye; DABCYL, 4-(4′-dimethylaminophenylazo) benzoic acid; TAMRA, carboxytetramethylrhodamine; MGB, minor groove binder.

ever, by using a BLASTP search (Schaffer et al., 2002) with the rat ABP protein sequence (AAC36313), we have detected a protein (KIAA1719) encoded by an uncharacterized human gene (BAB21810, AB051506), which has a high level of sequence similarity to the rat ABP (85% identity in the entire amino acid sequence and 92% identity in the sequence spanning the PDZ1–PDZ6 regions). This level of sequence conservation is sufficient to conclude that KIAA1719 is the likely human ABP ortholog. Accordingly, the sequence of the KIAA1719 mRNA (AB051506) was used to generate primers and probes for the human ABP gene. KIAA1719 was previously used as a possible ortholog of rat ABP in a study that described the mRNA expression of AMPAR-associated postsynaptic protein in macaque brain (Beneyto and Meador-Woodruff, 2004). PCR primers for amplification of AMPARs were designed so that they did not distinguish between any known splicing variants of the receptor subunits (Dingledine et al., 1999). Molecular beacons (MBs) were used as fluorogenic probes in the AMPARs real-time PCR assays. They were designed as described in our previous publication (Dracheva et al., 2001) and were synthesized commercially (IDT, Coralville, IA). TaqMan probes were used for SAP97, ABP, GRIP, and PICK1 mRNA quantitation; the primers and probes for these assays were designed in the Primer Express software program (Applied Biosystems, Foster City, CA) according to the manufacturer's recommendations and were synthesized by the same company. All PCR primers and fluorogenic probes that were used in the study are shown in Table II.

Real-time PCR. Real-time PCR analysis was performed with an ABI Prism 7700 Sequence Detector (Applied Biosystems). The MB PCR assays (GluR1–4 and β-actin) were carried out as described previously (Dracheva et al., 2001, 2003). The concentrations of probes were 0.07 μM β-actin MB; 0.17 μM GluR1, GluR2, and GluR4 MBs; and 0.3 μM GluR3 MB. Each 25-μl TaqMan PCR (SAP97, ABP, GRIP, PICK1, and β-actin) contained 5 μl of the relevant cDNA; 0.15 μM of a TaqMan probe; 300–400 nM of each primer; 1.25 units of Amplitaq Gold DNA polymerase; 0.25 unit of AmpErase UNG (both enzymes from Applied Biosystems); 200 μM each of dATP, dCTP, dGTP; 400 μM of dUTP; 4–5.5 mM $MgCl_2$; and 1× TaqMan buffer A (Applied Biosystems). The thermal cycling program consisted of 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. Negative control samples (no-cDNA) were run

for each assay; these samples rendered negligible amounts of amplification. To ensure the absence of amplification artifacts, PCR products for each real-time PCR assay were initially assessed on ethidium bromide-stained agarose gel. All assays used in the study rendered a single PCR product.

The reactions were quantitated by selecting the amplification cycle when the PCR product of interest was first detected (threshold cycle; Ct). Tests of assay linearity were conducted for all real-time PCR assays as previously described (Dracheva et al., 2001). To account for sample-to-sample variability, such as differences in RNA extractions and differences in the efficiencies of RT reactions, the expression level of each transcript (GluR1–4, SAP97, ABP, GRIP, PICK1) was normalized to the expression level of an endogenous reference (β-actin) in each sample. To account for differences between the runs, the expression levels for all targets were further normalized by expressing them as a ratio of the threshold cycle for the cDNA isolated from a pooled sample derived from combining cortical specimens from 10 randomly selected cases (calibrator). Pooled cDNA was run in every plate simultaneously with experimental samples. The relative expressions of GluR1–4, SAP97, ABP, GRIP, and PICK1 mRNA were calculated as $2^{-\Delta\Delta Ct}$ [the amount of target, normalized to an endogenous reference (β-actin) and relative to a calibrator (pooled sample)] as described by Livak and Schmittgen (2001). Only one cDNA was amplified in GluR1–4, ABP, GRIP, and β-actin PCRs (monoplex). SAP97 and PICK1 cDNAs were amplified in one tube (multiplex). The absence of competition between SAP97 and PICK1 PCRs was confirmed by comparing the amplification data obtained in the monoplex and multiple amplifications of these targets in serial dilutions (1:10–1:10,000) of the pooled cDNA (Bai et al., 2004). All samples were run in quadruplicate.

Despite the fact that real-time PCR is the most reliable quantitative method for nucleic acid analysis (Higuchi et al., 1993; Tyagi and Kramer, 1996; Bonnet et al., 1999), only relative quantitation of mRNA is possible with this method. The problem of absolute mRNA quantitation by PCR analysis is twofold. 1) Different PCR assays utilize unique primer/probe sets and, therefore, have different target amplification efficiencies, which are always less than 100%. This renders inaccurate any direct comparison of expression levels between different transcripts (e.g., GluR1 vs. GluR2) that are measured by independent PCRs. 2) PCR assays that are designed to quantitate mRNA expression utilize cDNA, which is transcribed from an RNA template in RT reactions. Accordingly, cDNA (not RNA) is analyzed in PCR assays. cDNA copies for the specific mRNA transcripts can be quantitated by using absolute standards prepared from plasmid DNA, which contains the transcript of interest as an insert (see below; see also Livak and Schmittgen, 2001). Standard curves are prepared for each target and for an endogenous reference (e.g., β-actin), and the experimental results are quantitated against these standard curves. The normalized values (the relative abundance) for different targets represent the expression values of their corresponding mRNAs and can be directly compared among each other.

To determine the relative abundances of GluR1–4, SAP97, ABP, GRIP, and PICK1 cDNA templates in experimental samples, PCR amplicons that were specific for these targets and for β-actin were subcloned into the vector (pCR-BluntII-TOPO), and the resultant plasmids (absolute standards) were used to obtain the standard curves. GluR1–4, SAP97, ABP, GRIP, PICK1, and β-actin DNA amplicons were synthesized in separate PCRs using *Pfu* Turbo DNA Polymerase (Stratagene, La Jolla, CA), the pooled sample cDNA (see above) as a template, and target specific primers (Table II). The PCR products were first resolved by agarose gel electrophoresis. The DNA was excised from a gel, purified by using a MinElute Gel Extraction Kit (Qiagen, Chatsworth, CA) and subcloned into pCR-BluntII-TOPO vector using a Zero Blunt TOPO PCR Cloning Kit (Invitrogen, La Jolla, CA). The inserts of the individual clones were confirmed by DNA sequencing (Elim Biopharmaceuticals, Hayward, CA). The concentrations of plasmids were determined by absorbance at 260 nm and converted to the number of copies by using the molecular weight of the vector and the inserts. The plasmid DNAs diluted 10^5–10^{11} were subjected to the real-time PCRs with the specific primers (Table II) to generate the standard curves for each reaction (Fig. 1; β-actin standard curve is not shown). Each sample was run in quadruplicate. For each experimental sample, the abundances of GluR1–4, SAP97, ABP, GRIP, PICK1, and β-actin cDNAs were calculated from the appropriate standard curve based on the Ct values determined for each sample in the real-time PCR measurements of mRNA expression described above. The normalized target values (the relative abundances) were obtained by dividing the target amount by the β-actin amount.

Statistical Tests of Significance

Analysis of covariance (ANCOVA) followed by Newman-Keuls tests and *t*-tests was used to analyze the results of these studies. ANCOVA was used for analyses based on the entire cohort, where the age of the subject at the time of death was entered as the covariate. *t*-Tests were used to compare differences between groups, when the groups had been matched for age at the time of death. Because each brain region and each target molecule were measured independently with different probes and in different experiments, differences between groups for the expression of each gene were assessed with independent tests of significance. Pearson product-moment correlation procedures were used to assess the correlation between the various continuously distributed variables. Statistical analyses were performed in Statistica for Windows (release 6.0; Statsoft Inc., Tulsa, OK) or SPSS for Windows (version 12; SPSS Inc., Chicago, IL).

RESULTS

Relative mRNA Expression

The expression of GluR1–4, SAP97, ABP, PICK, and GRIP did not correlate significantly with postmortem interval or with tissue pH ($rs < 0.26$, $Ps > 0.09$). Depending on the specific transcript and brain region, however, age at death correlated negatively with the abundance of these transcripts (rs range from −0.19 to −0.41, Ps range from 0.17 to 0.001). Therefore, age was entered as a covariate in analyses of variance. Results were also analyzed in subgroups of cases matched in age to

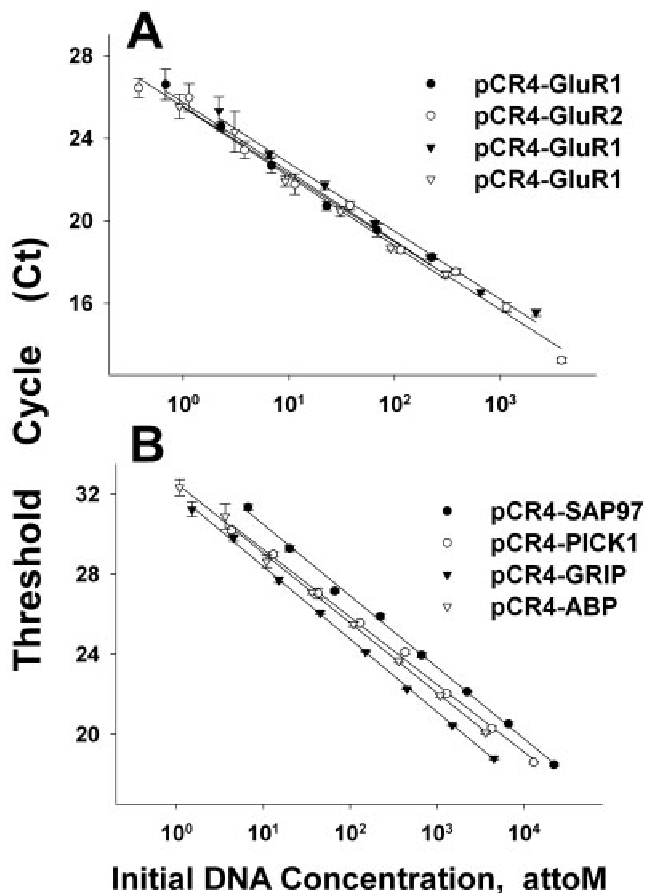

Fig. 1. Absolute standard curves obtained with recombinant plasmid DNAs containing GluR1–4 (**A**) or AMPAR binding protein (**B**) amplicons as inserts. The plasmid DNAs were diluted 10^6–10^{10} times to be at concentrations similar to those of the target molecules in the experimental samples and were subjected to real-time PCRs using primers specific for each target (see Table II). Each sample was run in quadruplicate. Values represent mean ± SD.

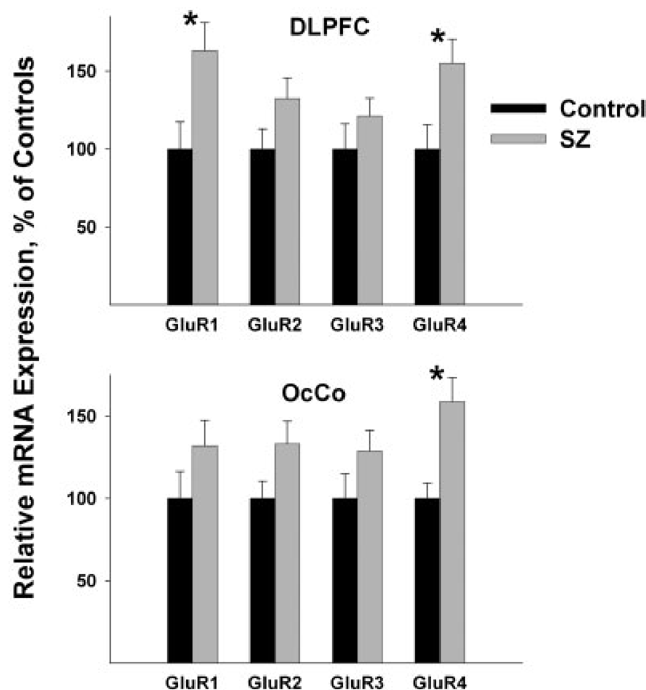

Fig. 2. Relative expression of the AMPAR subunit (GluR1–4) mRNAs in the DLPFC and the occipital cortex (OcCo) of normal controls (N = 26) and schizophrenics (SZ; N = 36). The relative mRNA expression for each target was measured by real-time PCR and was calculated as $2^{-\Delta\Delta Ct}$ [the amount of target, normalized to an endogenous reference (β-actin) and relative to a calibrator (cDNA from a pooled sample)], as described in Materials and Methods. Values represent mean ± SEM. Asterisks indicate statistically significant differences between control and schizophrenia groups.

within 1 year of each other. In addition, the sex distribution within the schizophrenia and control groups was different ($\chi^2 = 6.8$, $P = 0.01$). To control for sex differences, sex was also entered as a covariate in the analyses of variance.

The relative mRNA expression of GluR1 and GluR4 subunits was found to be significantly [F(1,58) > 5.04, *P*s < 0.03] increased in the DLPFC of schizophrenics relative to normal controls. In addition, mRNA expression of the GluR4 was elevated in the occipital cortex of the schizophrenics [F(1,58) = 7.0, *P* = 0.01]. No statistically significant changes were detected in the expression of GluR1 in the occipital cortex or in the expression GluR2 and GluR3 in either cortical area (Fig. 2); however, the expression of all AMPAR subunits in both cortical areas was nominally elevated in persons with schizophrenia. In addition, similar results were obtained when subjects within the schizophrenia and control groups were matched for PMI to within 60 min of each other (Ns = 17 and 21, respectively, *P*s < 0.03). Furthermore, when subjects within diagnostic groups were matched for sex, differences between groups persisted for males (Ns = 11 and 24 for controls and schizophrenics, respectively, *P*s < 0.06), however, in the smaller group of females (Ns = 15 and 8 for controls and schizophrenics), only the increased expression of GluR1 in the DLPFC and occipital cortex reached statistical significance (*P*s < 0.06).

Relative mRNA expression of GRIP and ABP was increased in the occipital cortex of schizophrenics [F(1,58) > 5.5, *P*s < 0.02]. GRIP mRNA level was also elevated in the DLPFC of schizophrenia patients [F(1,58) = 4.17, *P* = 0.05], whereas ABP mRNA was only nominally and statistically nonsignificantly increased in this area. The mRNA expressions of SAP97 and PICK1 were not changed in schizophrenia (Fig. 3). Similar to the findings for GluR subunits, differences in the expression of AMPAR binding proteins persisted (all *P*s < 0.06) when groups were matched for PMI or for sex, except for the expression of GRIP in males, which did not reach statistical significance.

Although the data reported above were analyzed by using ANCOVA where age at death was entered as a

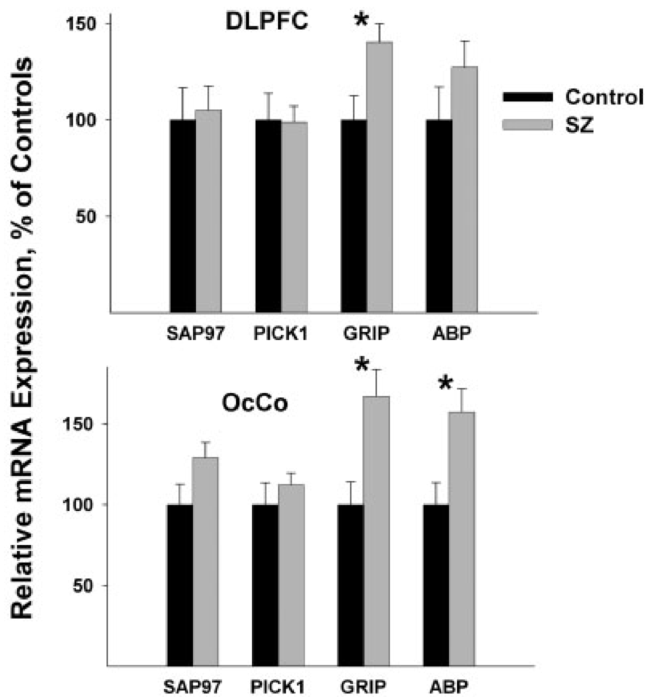

Fig. 3. Relative expression of AMPAR binding proteins (SAP97, PICK1, GRIP, and ABP) mRNAs in the DLPFC and the occipital cortex (OcCo) of normal controls and schizophrenics. See Figure 2 legend for details.

covariate, the data were also analyzed in subsets of 20 and 17 cases of controls and schizophrenics, respectively, where cases and controls were selected to match in age to within 12 months of each other. *t*-Test of GluR1, GluR4, GRIP, and ABP expression in the brain regions where significant differences had been found by ANCOVA confirmed the statistically significant elevations in gene expression for these transcripts (ts < 2.06, Ps < 0.05) even when groups were matched for age (not shown).

Relative mRNA Abundances

The relative abundances of GluR1–4, SAP97, ABP, GRIP, and PICK1 mRNAs in each sample were calculated from the appropriate standard curves (Fig. 1) as described in Materials and Methods. As expected, these calculated amounts showed the same differences between the schizophrenia and the normal control groups that were observed for relative mRNA expression values described above (Table III).

The relative mRNA abundances of different genes can be directly compared with each other when the quantitative methods described above are used. There were significant differences in mRNA abundances between different AMPAR subunits and between different AMPAR binding proteins in both control and schizophrenia groups [repeated-measures ANOVA $F(3,177) = 59.9$, $P = 0.00001$; Table III, Fig. 4]. These differences were similar in the schizophrenia and control groups. The relative abundances of GluR1, GluR2, GluR3, and GluR4 mRNAs (calculated as a percentage of total amount of AMPAR subunits) were 7.5% ± 0.4%, 64.2% ± 1.0%, 24.2% ± 0.8%, and 4.5% ± 0.2%, respectively, in the occipital cortex (N = 62) and 8.3% ± 0.5%, 58.6% ± 1.1%, 29% ± 0.8%, and 4.4% ± 0.4%, respectively, in the DLPFC (N = 61) of the entire cohort. GluR2 and GluR3 were the major components in the AMPAR subunits expressed in both cortical regions. With calculation as a percentage of the total amount of AMPA subunits, significant differences in the amounts of GluR2 and GluR3 mRNAs were detected between the two cortical areas ($P < 0.0003$). More GluR2 and less GluR3 mRNAs were expressed in occipital cortex than in the DLPFC (Fig. 4A). The relative abundances of SAP97, PICK1, GRIP, and ABP mRNAs were 87.6% ± 0.5%, 7.3% ± 0.3%, 3.5% ± 0.3%, and 1.7% ± 0.1%, respectively, in the occipital cortex (N = 61) and 86.6% ± 0.5%, 7.1% ± 0.3%, 4.6% ± 0.3%, and 2.0% ± 0.1%, respectively, in the DLPFC (N = 61) of the entire cohort. More GRIP mRNA was detected in the DLPFC than in the occipital cortex ($P = 0.017$; Fig. 4B).

Although all schizophrenia subjects had been exposed to neuroleptic medications during the course of their illness, antipsychotic medications had been discontinued for some for different amounts of time prior to death, ranging from 0 to 246 weeks. To determine whether the expression of AMPARs and AMPAR binding proteins was influenced by antipsychotic medications received proximal to death, Pearson's correlation coefficients were calculated for the abundance of each mRNA under study and the number of weeks off neuroleptics. All rs were less than −0.19, and none of the correlations reached statistical significance (Ps > 0.31). As a further check on the influence of neuroleptics on AMPAR and AMPAR binding proteins, the schizophrenia group was stratified into those who were continuously exposed to antipsychotic medications and those who were exposure free for 6 weeks or more [i.e., 6 half-lives of haloperidol in human brain after chronic treatment (Kornhuber et al., 1999)]. This analysis showed that the two groups were statistically indistinguishable from each other with respect to the expression of AMPARs and AMPAR binding proteins except for the lower expression of GluR1 in "drug-free" cases in the DLPFC (Ns = 23 and 12 for drug exposed vs. "drug free" schizophrenics, respectively; $P < 0.006$).

DISCUSSION

The results of this study showed a significant increase in the expression of mRNAs encoding two subunits of the AMPAR (GluR1 and GluR4) and two of the four studied AMPAR binding proteins (ABP and GRIP) in the cerebral cortex of schizophrenia subjects. The observed changes were region specific, insofar as GluR1 was significantly increased only on the DLPFC and ABP only in the occipital cortex, whereas GluR4 and GRIP were significantly changed in both regions studied. However, nominal increases in mRNA expression of all GluR subunits and ABP were observed in the both cortical areas. These data point to a possible abnormality in the AMPAR system and, by inference, in glutamatergic functioning in schizo-

TABLE III. Relative Abundances of the AMPAR Subunits and AMPAR Binding Proteins mRNAs in the DLPFC (PFC) and the Occipital Cortex (OcCo) of the Subjects With Schizophrenia (SZ) and the Normal Controls (NC)†

		AMPAR subunits								AMPAR binding proteins							
		GluR1		GluR2		GluR3		GluR4		SAP97		PICK1		GRIP		ABP	
		PFC	OcCo	PFC	OcCo	PFC	OcCo	PFC	OcCo	PFC	OcCo	PFC	OcCo	PFC	OcCo	PFC	OcCo
NC (N = 26)	Mean	0.31*	0.41	2.42	3.23	1.36	1.22	0.15*	0.18*	4.70	4.85	0.39	0.39	0.17*	0.15*	0.10	0.08*
	SEM	0.06	0.07	0.33	0.35	0.25	0.20	0.02	0.02	0.54	0.58	0.06	0.04	0.02	0.02	0.02	0.01
SZ (N = 36)	Mean	0.48*	0.54	3.21	4.32	1.61	1.56	0.22*	0.29*	5.55	6.00	0.43	0.45	0.26*	0.24*	0.13	0.13*
	SEM	0.06	0.06	0.36	0.45	0.17	0.15	0.02	0.03	0.63	0.39	0.06	0.03	0.02	0.02	0.01	0.01

†For each experimental sample, the amounts of GluR1–4, SAP97, ABP, GRIP, PICK1, and β-actin cDNAs were calculated from the appropriate standard curve based on the Ct values determined for each sample in the real-time PCR measurements of mRNA expression. The relative abundances were calculated by dividing the target amount by the β-actin amount. Values represent mean ± SEM.
*Statistically significant differences between control and schizophrenia cohorts.

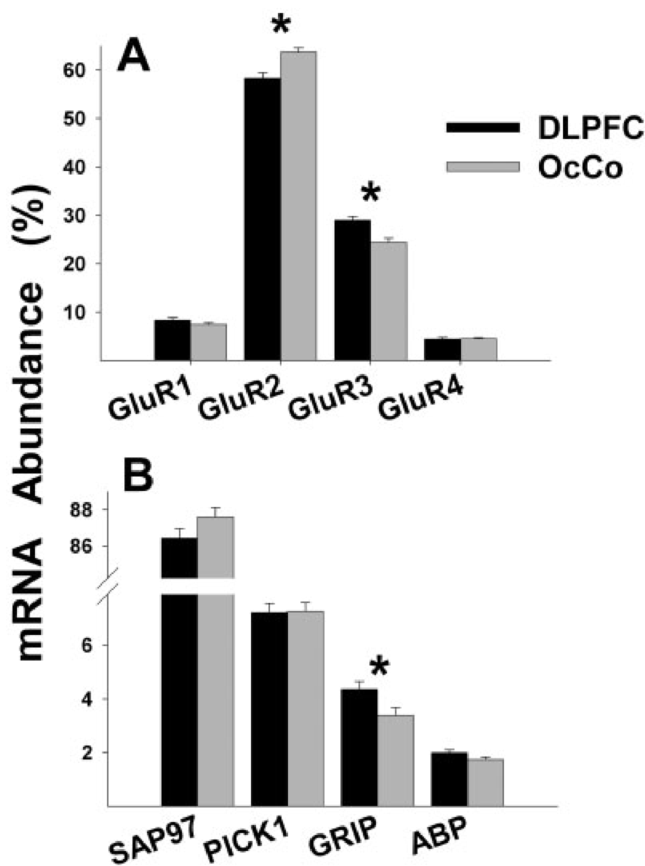

Fig. 4. Relative abundances of GluR1–4 (**A**) and AMPAR binding proteins (**B**) in the DLPFC and the occipital cortex of the entire cohort. The values are expressed as a percentage of total amount of AMPAR subunits (A) or of total amount of AMPAR binding proteins (B) and represent mean ± SEM. Asterisks indicate statistically significant differences between the DLPFC and the occipital cortex. Significant differences in the amounts of GluR2, GluR3 (A), and GRIP1 (B) mRNAs were detected between the two cortical areas. Note that GluR2, GluR3 (A), and SAP97 (B) were the major components in the AMPAR subunits and AMPAR binding proteins, respectively.

phrenia. These results should be interpreted with caution, however, because, as is often the case in schizophrenia research, it is possible (see below) that at least some of the abnormalities in the AMPA receptor system described could have resulted from prolonged exposure to antipsychotic medications.

This is the first study in which the relative abundances of mRNAs that encode four AMPAR subunits (GluR1–4) and four AMPAR binding proteins (SAP97, PICK1, ABP, and GRIP) were measured in two different human cortical regions (DLPFC and occipital cortex). The relative abundances of GluR1–4 subunits mRNA in the human DLPFC and occipital cortex were GluR2 > GluR3 > GluR1 > GluR4. GluR2 (58–64%) and GluR3 (24–29%) were the main components of the cortical human AMPARs; therefore, the major AMPAR complexes in the human cortex are probably those containing GluR2 and GluR3 subunits. Although to our knowledge there have been no studies that specifically investigated the abundances of AMPAR subunits in human cortical tissues, our data are in agreement with reports that, in the rat somatosensory cortex, the majority of neurons (approximately two-thirds) showed expression of the GluR2 and undetectable expression of the GluR1 subunit (Kondo et al., 1997). In contrast, cultured rat hippocampal pyramidal neurons express high levels of GluR1 and GluR2 mRNAs (~48% and 45%, respectively; Tsuzuki et al., 2001), and one of the major types of AMPAR complexes that are present in the rat CA1/CA2 hippocampal neurons is those made up of GluR1 and GluR2 subunits (Wenthold et al., 1996).

The properties of AMPAR depend mostly on the presence of the GluR2 subunit, which determines both the rectification properties and the calcium permeability of the receptor channels (Hollmann et al., 1991; Geiger et al., 1995; Jonas and Burnashev, 1995). AMPARs with GluR2 subunits display a linear or outward rectification and little calcium permeability. In contrast, receptors lacking this subunit exhibit strong inward rectification and high calcium permeability. Most neurons express GluR2 and form calcium-impermeable channels, but populations of neurons that lack GluR2 have been identified in both hippocampus

and cortex (Bochet et al., 1994; Wenthold et al., 1996; Kondo et al., 1997). Although the influence of the other subunits is less dramatic, the properties of the receptor are dependent on the subunit and splice variant composition (Mosbacher et al., 1994; Geiger et al., 1995). Thus, differential expression of GluR subunits could provide AMPARs in the CNS with functional diversity. Therefore, the small differences detected in the expression of GluR2 and GluR3 genes between DLPFC and occipital cortex (more GluR2 and less GluR3 mRNAs were found in the occipital cortex than in the DLPFC) may indicate the presence of subtle differences in the functionality of the glutamatergic system between these two cortical areas.

The expression of SAP97 mRNA in DLPFC and occipital cortex was significantly higher (by over 10 times) than the expression of other genes coding for other AMPAR binding proteins investigated (PICK1, GRIP, and ABP). SAP97 is believed to be involved in the synaptic trafficking of GluR1 subunit (Sans et al., 2001; Rumbaugh et al., 2003). At least in vitro, SAP97 can bind to numerous neuronal proteins in addition to GluR1, including NMDAR NR2 subunits (Niethammer et al., 1996; Gardoni et al., 2003), K^+ channels (Tiffany et al., 2000), and multiple other MAGUKs (Karnak et al., 2002). A recent study demonstrated that RNAi knockdown of endogenous SAP97 in hippocampal neurons reduced surface expression of both GluR1 and GluR2 and inhibited both AMPA- and NMDA-mediated excitatory postsynaptic currents (EPSCs), implying additional roles of SAP97 in maintaining the functional and morphological integrity of synapses (Nakagawa et al., 2004). Also, in contrast to other MAGUKs and AMPAR binding proteins, SAP97 is expressed in nonneuronal cells (Muller et al., 1995). In epithelial cells, SAP97 has been found localized at sites of cell–cell contact along the lateral membrane, where they are integrated into the cortical cytoskeleton, suggesting that SAP97 may play a scaffolding role in the assembly and structural integrity of various types of membrane specialization (Reuver and Garner, 1998). Thus, high expression of SAP97 message can be explained by the diversity of roles of SAP97 in cellular function.

The relative mRNA expressions of GluR1 and GluR4 subunits and GRIP was significantly increased in the DLPFC of schizophrenics relative to normal controls. In addition, mRNA expression of GluR4, GRIP, and ABP was also elevated in the occipital cortex of the schizophrenics. In rodents and nonhuman primates, populations of neurons expressing primarily GluR1 have been identified in the cortex, striatum, and spinal cord, and such neurons have been hypothesized to form predominantly homomeric receptors (Petralia and Wenthold, 1992; Martin et al., 1993; Tachibana et al., 1994). In a hippocampal culture study, a few nonpyramidal neurons exhibited a strong inward rectification and a high calcium permeability (Iino et al., 1990), and single-cell RT-PCR and patch-clamp studies revealed that they express only two subunits, GluR1 and GluR4 (Bochet et al., 1994). In the rat somatosensory cortex, the neurons that contain GluR1 but have undetectable GluR2 subunit immunostaining constituted 10–15% of cortical neurons and had mostly nonpyramidal shapes (Kondo et al., 1997). Most of these neurons contained GluR4 subunits and were parvalbumin immunoreactive (Kondo et al., 1997). Because parvalbumin is found almost exclusively in GABA neurons in the cerebral cortex (Celio, 1986; van Brederode et al., 1991; Ren et al., 1992), these GluR1-expressing neurons are likely to be GABAergic. A recent immunohistochemical study of AMPAR binding proteins in the rat cerebral cortex has demonstrated that most GRIP-positive cells also contained ABP, and vice versa. However, cells strongly immunopositive for ABP (mainly pyramidal neurons) were weakly stained for GRIP, whereas cells strongly immunopositive for GRIP (a subset of nonpyramidal neurons) were weakly stained for ABP (Burette et al., 2001). This study also identified a small group of ABP-intense, GluR2/3-negative cortical cells (Burette et al., 2001). Based on their morphology, location, and intense staining for GRIP, the authors surmised that these were likely to represent a specialized population of GluR1-rich GABAergic interneurons (Burette et al., 1999), suggesting that ABP and GRIP play some scaffolding role unrelated to GluR2/3 in these neurons. These observations and the data reported here support the hypothesis of increased expression of GluR1/GluR4 containing AMPARs and AMPAR binding proteins (GRIP and ABP) in this specific subset of cortical local inhibitory GABAergic neurons in elderly schizophrenics. Alternatively, the number of these specific GABAnergic neurons might be increased in schizophrenia patients. Consistent with either possibility, we recently demonstrated, with the same experimental cohort, enhanced mRNA expression of major GABA-synthesizing enzymes (GAD67 and GAD65) in the DLPFC and occipital cortex of schizophrenia patients in comparison with matched normal controls (Dracheva et al., 2004).

As was mentioned above, increased ligand binding to AMPARs has recently been demonstrated in cortices of schizophrenics (Noga et al., 2001; Zavitsanou et al., 2002). Our study indicated significant increases in GluR1 and GluR4 mRNA expression and nominal increases in the expression of GluR2 and GluR3 subunits in two cortical areas of schizophrenia patients. With a slightly larger sample, or less variance, the increases detected for the two statistically "unchanged" major subunits of AMPAR (GluR2 and -3) would likely also attain statistical significance. Therefore, it is plausible that the overall elevation in the AMPAR neurotransmission could be a characteristic of the disease.

Possible alterations in expression of AMPAR and AMPAR binding proteins in schizophrenia have been extensively investigated in a number of cortical regions (Breese et al., 1995; Healy et al., 1998; Sokolov, 1998; Noga et al., 2001; Toyooka et al., 2002; Zavitsanou et al., 2002). The results of these studies, however, are markedly discordant (Meador-Woodruff and Healy, 2000). There are several possible reasons for these inconsistencies, including differences in

methodology and in cortical regions investigated. The most parsimonious explanation for the discrepancy lies in the characteristics of the cohorts selected in the different studies. Differences in the severity of disease, diagnostic certainty, subjects' age at death, cause of death, PMIs, drug regimens, etc., might possibly contribute to the outcomes of the studies. The methods employed in the current study aimed to address many of these issues and possible sources of experimental error. The study reported here was performed on a cohort of well-characterized and antemortem assessed and diagnosed elderly patients with schizophrenia and matched normal controls (Davidson et al., 1995; Davidson and Haroutunian, 1995; Harvey et al., 1998). The real-time PCR methodology used for mRNA expression measurements is arguably the most advanced and quantitative method for DNA and RNA quantitation (Higuchi et al., 1993; Tyagi and Kramer, 1996; Bonnet et al., 1999). The use of gene-specific probes in contrast to nonspecific approaches, such as SYBR green, added to the fidelity of the assay system used. In addition, the linearity and fidelity of each PCR assay were carefully optimized for the full range of individual differences in mRNA expression between the samples.

Among the issues of concern in almost every postmortem neurobiological study of schizophrenia is the influence of antipsychotic medications on the variables under investigation. The potential influence of antipsychotic medications was analyzed by determining whether the number of weeks prior to death for which subjects had been free of neuroleptic medications (0–246 weeks) correlated with the abundance of the mRNAs under study. Significant correlations between the abundance of any of the AMPAR subunits or binding proteins were not observed. This conclusion was supported in part by the lack of differences between "drug-free" and drug-exposed schizophrenics for the majority of the GluRs and AMPAR binding proteins investigated. The failure to find significant correlations between time free of neuroleptic medications and AMPARs and their associated intracellular machinery does not preclude a more permanent influence of antipsychotic medications, however. Indeed, comparison of "drug-free" and drug-exposed schizophrenics showed that the expression of GluR1 in the DLPFC was significantly reduced in the former group. Whether neuroleptic exposure had a true influence on GluR1 gene expression or whether the apparent increase in GluR1 expression in the drug-exposed group was a spurious finding resulting from the smaller sample sizes analyzed remains to be determined. Although it is often impossible to address fully the role of lifetime exposure to antipsychotic medications, studies in chronically neuroleptic-exposed animals provide one avenue of exploration. Unfortunately, numerous experiments on the possible influence of neuroleptics on the expression of AMPARs have yielded inconsistent results (Oretti et al., 1994; Fitzgerald et al., 1995a; Eastwood et al., 1996; Meador-Woodruff et al., 1996; Tarazi et al., 1996; McCoy et al., 1998). Oretti et al. (1994) found no change in whole-rat-brain RNA levels of any of the AMPAR subunit mRNAs after 1–32 days of treatment with a variety of antipsychotics. Also, GluR1 mRNAs were found to be entirely unaffected in rat forebrain by 16 days of haloperidol treatment (Eastwood et al., 1996). However, consistent with the results described above, immunoblotting was used to show that 30 days of haloperidol treatment increased GluR1 protein abundance in the medial prefrontal cortex of rats (Fitzgerald et al., 1995b). Up-regulation of hippocampal GluR1 mRNA by haloperidol has also been reported (Meador-Woodruff et al., 1996). In another study, however, subchronic and chronic antipsychotic drug treatment did not alter [^{3}H]CNQX (AMPA) binding in the rat medial prefrontal cortex (Tarazi et al., 1996). Two recent studies reported decreased [^{3}H]CNQX (AMPA) receptor binding in the prefrontal cortex and putamen of nonschizophrenic neuroleptic-treated normal subjects compared with nontreated controls (Noga et al., 1997, 2001). Thus, when these results and those reported here are viewed in their totality, it is not obvious which changes in AMPARs and AMPAR binding proteins are attributable to schizophrenia and which to the medications used to treat it. Either outcome would be of significant therapeutic and expository interest. If the changes in the AMPA receptor system are attributable to antipsychotic medications, then they raise the possibility that the observed up-regulation may be important to therapeutic response. If the changes in gene expression are intrinsic to the disease process itself, then they suggest that the dysregulation of AMPA receptor systems and their associated proteins is part of the disease process and point to novel directions for future experimentation.

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