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Differential Regulation of α7 Nicotinic Receptor Gene (*CHRNA7*) Expression in Schizophrenic Smokers

Sharon Mexal[3], **Ralph Berger**[1], **Judy Logel**[1], **Randal G. Ross**[1], **Robert Freedman**[1,2], and **Sherry Leonard**[1,2]

Sharon Mexal: ; Ralph Berger: ; Judy Logel: ; Randal G. Ross: ; Robert Freedman: ; Sherry Leonard: Sherry.Leonard@ucdenver.edu

[1] Department of Psychiatry, University of Colorado Denver, Mail Stop 8344, P.O. Box 6511, Aurora, CO 80045, USA

[2] The Veterans Affairs Medical Research Service, Denver, CO, USA

[3] Cenomed BioSciences, Irvine, CA, USA

Abstract

The α7 neuronal nicotinic receptor gene (*CHRNA7*) has been implicated in the pathophysiology of schizophrenia by genetic and pharmacological studies. Expression of the α7* receptor, as measured by [^{125}I]α-bungarotoxin autoradiography, is decreased in postmortem brain of schizophrenic subjects compared to non-mentally ill controls. Most schizophrenic patients are heavy smokers, with high levels of serum cotinine. Smoking changes the expression of multiple genes and differentially regulates gene expression in schizophrenic hippocampus. We examined the effects of smoking on *CHRNA7* expression in the same tissue and find that smoking differentially regulates expression of both mRNA and protein for this gene. *CHRNA7* mRNA and protein levels are significantly lower in schizophrenic nonsmokers compared to control nonsmokers and are brought to control levels in schizophrenic smokers. Sufficient protein but low surface expression of the α7* receptor, seen in the autoradiographic studies, suggests aberrant assembly or trafficking of the receptor.

Keywords

Nicotinic receptor; Schizophrenia; Smoking; Gene expression; α7; CHRNA7

Introduction

The development of schizophrenia is thought to involve the interaction of multiple genetic, epigenetic, and environmental factors (Harrison and Weinberger 2005). One of the candidate genes for schizophrenia, the α7 neuronal nicotinic acetylcholine receptor gene, *CHRNA7*, and its holoreceptor, α7*, may also contribute to the high incidence of smoking in this population. The α7* receptor is a member of a large gene family that includes 14 known genes expressed in the central nervous system and in the periphery (Leonard and Bertrand 2001; Lindstrom 1997). Receptor subunits assemble as pentamers (Cooper et al. 1991) in the endoplasmic reticulum and migrate through the golgi to the cell surface (Cooper and Millar 1997; Green and Millar 1995; Wanamaker et al. 2003). The α7* receptor is thought to be a homopentamer that binds nicotine with low affinity and binds the snake toxin, α-bungarotoxin, with high

Correspondence to: Sherry Leonard, Sherry.Leonard@ucdenver.edu.

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affinity (Wonnacott 1986; Wonnacott et al. 2005). Activation by the endogenous ligand, acetylcholine, or nicotine in tobacco products results in channel opening and increased intracellular Ca^{++} (Vijayaraghavan et al. 1992). Presynaptically, Ca^{++} influx leads to neurotransmitter release (Grady et al. 1992; Guo et al. 1998; Jones and Wonnacott 2004; Kaiser and Wonnacott 2000; MacDermott et al. 1999; Rousseau et al. 2005; Wonnacott 1997). Localization and Ca^{++} influx at postsynaptic sites, likely in or near postsynaptic densities, can lead to changes in gene expression (Albuquerque et al. 1998; Levy and Aoki 2002; Maggi et al. 2003; Mexal et al. 2005; Shoop et al. 1999).

The *CHRNA7* gene has been implicated in cognitive and sensory deficits in schizophrenia by both genetic and pharmacological studies. Schizophrenics have several types of sensory processing deficits, including auditory sensory processing (Adler et al. 1991; Freedman et al. 1999) and eye-tracking abnormalities (Holzman et al. 1973; Levy et al. 1993; Ross et al. 1998). Both of these deficits are normalized by nicotine, delivered as gum, or by smoking (Adler et al. 1993; Olincy et al. 1998; Olincy et al. 2003). In animal models of the P50 auditory sensory processing deficit, nicotine and specific agonists of the α7* receptor normalize the deficit (Simosky et al. 2001; Stevens et al. 1998). Nicotine also improves cognitive deficits in schizophrenia (George et al. 2002; Levin et al. 1999, 2006; Weiser et al. 2004), and the α7* receptor has been identified as an important target for the development of drugs for cognitive deficits in the disorder (Kuehn 2006). Indeed, Phase I and II trials of the *CHRNA7* partial agonist, DMXB-A, found significant improvement in attention in nonsmoking schizophrenics (Freedman et al. 2008; Olincy et al. 2006). The locus of the *CHRNA7* gene has been genetically linked to the sensory processing deficit in schizophrenia (Freedman et al. 1997) and to the disease in multiple studies (Abecasis and Cookson 2000; Freedman et al. 2001; Kaufmann et al. 1998; Leonard et al. 1998; Liu et al. 2001; Riley et al. 2000; Stöber et al. 2000; Tsuang et al. 2001; Xu et al. 2001). It is also linked to smoking in the disorder (De Luca et al. 2004).

We have previously screened the coding region and proximal promoter of the *CHRNA7* gene in control and schizophrenic subjects. We did not find mutations in the coding region that were associated with schizophrenia (Gault et al. 2003). However, we did isolate mutations in the proximal promoter region that were associated with both schizophrenia and the P50 sensory processing deficit (Leonard et al. 2002). Many of these mutations functionally decrease transcription in a reporter gene assay.

The prevalence of smoking in schizophrenia is inordinately high. More than 80% of schizophrenics smoke compared to 25% of the general population (Dalack et al. 1998; de Leon and Diaz 2005; George and Krystal 2000; Kumari and Postma 2005). Schizophrenics smoke high-tar cigarettes and extract more nicotine per cigarette than do normal smokers (Olincy et al. 1997).

The expression of the α7* receptor, as measured by [^{125}I]-α-bungarotoxin binding, is decreased by approximately 50% in postmortem hippocampus of schizophrenic subjects (Freedman et al. 1995). Receptor expression is also reduced in cortex (Guan et al. 1999; Marutle et al. 2001) and in the reticular nucleus of the thalamus (Court et al. 1999). These data suggest that schizophrenics may be smoking to self-medicate some of the underlying pathology in the disorder (Adler et al. 1998; Leonard 2003; Leonard et al. 2007).

We have recently found that smoking changes gene expression for more than 250 genes in postmortem hippocampus (Mexal et al. 2005). Some of the expression changes may be long lasting (Hope et al. 2007). In the same microarray study, we found that many genes were differentially regulated by smoking in schizophrenic subjects. The pattern was generally the same. Expression was either increased or decreased in schizophrenic nonsmokers compared to control nonsmokers and at control levels in schizophrenic smokers. One of these genes,

differentially regulated by smoking in schizophrenia, was the *CHRNA7* gene. We have examined the relationship between mRNA and protein expression for the *CHRNA7* gene in postmortem hippocampus of schizophrenic and control smokers and nonsmokers, finding that expression is differentially regulated in schizophrenic subjects. Both mRNA and protein are increased in schizophrenic smokers compared to controls. *CHRNA7* promoter polymorphisms that decrease transcription in an in vitro reporter gene assay were found more frequently in schizophrenic nonsmokers with low levels of mRNA in hippocampus.

Materials and Methods

Human Postmortem Brain Collection

The Schizophrenia Research Center Brain Bank at the VA Medical Center in Denver, CO, provided postmortem brain tissue. Human brain was collected at autopsy from local services following family consent. At autopsy, the brain was weighed and examined for gross pathology. It was then divided sagittally, and one hemisphere, selected randomly, was preserved in formalin for neuropathological analysis at macroscopic and microscopic levels. Microscopic evaluations included standard Bielchowsky silver stain on multiple cerebral areas to rule out abnormal neuropathology, such as plaques and tangles, associated with Alzheimer's and other conditions. Patients with positive neuritic findings or ambiguous neuropathology reports were excluded from the current study. The hemisphere that was not subjected to neuropathological analysis was sliced coronally into 1-cm slices. Multiple regions were dissected in 1-g blocks, frozen in dry ice snow, and packaged for storage at −80°C.

Hippocampal tissue for the microarray study was derived from coronal Section 5. Each sample included CA4, CA3, CA2, CA1, and subiculum. Brain hemisphere, left or right, was randomly collected. Freeze delay, the time from brain removal to storage of the dissected tissue at −80° C, was always less than 2.5 h (Leonard et al. 1993). Brain pH measurement and RNA and protein isolation were carried out as previously described (Mexal et al. 2005, 2006).

Subjects

Subjects included in the mutation screening study were ascertained in the Denver Schizophrenia Center. DNA samples were isolated by standard methods from lymphoblasts or postmortem brain (Sambrook and Russell 2001).

Subjects included in the postmortem brain studies were selected after a review of records detailing pre- and postmortem parameters. This information was collected from an extensive review of hospital, autopsy, and neuropathology reports, as well as structured interviews with physicians and family, as previously described (Mexal et al. 2005). Briefly, subjects with neuropathological abnormalities, heavy alcohol use (>10 drinks per week), or illicit drug use were excluded from the study. Schizophrenics were selected using the narrow diagnostic criteria defined by the DSM-IV. Based on the records described above, two psychiatrists (RGR and RF) independently confirmed each patient's schizophrenia diagnosis and verified that controls had no history of mental illness. Patient records were also used to identify smokers and nonsmokers for this study. These records indicated packs of cigarettes smoked per day and pack years. A subject was included as a smoker in this study if that individual had been smoking any amount of cigarettes at the time of death. Nonsmokers included in the postmortem expression studies were selected if records indicated no history of smoking in the past 10 years. Several former smokers met this criterion and were, therefore, classified as nonsmokers in this study. Only never smokers were included in the mutation screening study.

Among the living subjects, controls were interviewed and found to have no evidence for current or past psychosis, using a Structured Clinical Interview for DSM-IV Axis I Disorders—Non-

Patient Edition (First et al. 1996). They also received a Family History-Research Diagnostic Criteria interview (Endicott J. et al. 1978). Living schizophrenics were interviewed and diagnosis confirmed using the narrow diagnostic criteria defined by the DSM-IV (American Psychiatric Association 1994).

Ethnicities of all subjects were recorded from self-report or family interview. The majority of the postmortem brain subjects included in the array analysis were Caucasian (91.2%). In order to match our expanded dataset utilized in our genotyping study to the postmortem brain samples, the majority of subjects included in this analysis were also Caucasian (approximately 99%). Three types of analysis were done in the current study: Microarray, real-time quantitative PCR (QRT-PCR), and western blot. A summary of the demographics on samples included in each analysis are shown in Table 1.

QRT-PCR

Total RNA was isolated and integrity verified as described (Leonard et al. 2005; Mexal et al. 2005, 2006, 2007). Single-strand cDNA was synthesized from total RNA (2 μg) using random hexamer primers and the Superscript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). To limit amplification from genomic DNA, primer sets for each gene were designed to cross intron/exon boundaries (OligoToolkit, http://oligos.qiagen.com/oligos/toolkit.php). BLAST searches were performed to confirm primer specificity. Primer sequences are listed in Table 2.

PCR amplification of cDNA was performed using 500 nM of primers and the Quantitect SYBR Green PCR Kit (Qiagen, Valencia, CA). PCR cycling conditions were 50°C for 2 min, 95°C for 15 min, then 40 cycles of 94°C for 15 s, 58°C for 30 s, and 72°C for 30 s. Formation of PCR products was monitored using an iCycler detection system (BioRad, Hercules, CA). Linear regression was performed by the iCycler software (BioRad) to extrapolate mRNA amounts in nanograms from a standard curve. Values were normalized to the GAPDH mRNA levels. For each experiment, a tube without the addition of reverse transcriptase enzyme or without the addition of cDNA was included to assess amplification from genomic DNA and nonspecific product formation, respectively. A melt curve analysis was also conducted to examine the uniformity of product formation, primer–dimer formation, and amplification of nonspecific products.

CHRNA7 Promoter Polymorphism Screening

CHRNA7 promoter polymorphisms were screened, utilizing DFPLC technology (WAVE™, Transgenomic, Inc., San Jose, CA). For the proximal promoter polymorphism screening, a 271-base pair PCR product was amplified using a primer set spanning −269 to +2 of the gene (Table 2). Primers (600 nM) and DNA (500 ng) were combined with the GC-RICH PCR System (Roche Diagnostics, Indian-apolis, Ind). PCR amplification was performed in a Gene-Amp PCR System 9600 (Perkin-Elmer) with the following conditions: 95°C, 3 min; 95°C, 30 s; 60°C, 30 s; 72°C, 30 s, 38 cycles; 4°C hold. An aliquot of the PCR fragment was used for heteroduplex formation in the thermal cycler as follows: 95°C for 5 min, ramp from 95°C to 25°C over 45 min, hold at 4°C. A melting profile for the fragment was determined with the Wavemaker program (Transgenomic, Inc). The resulting chromatograms showed the presence of heteroduplex peaks that were resolved optimally at 70.2°C. A triethylammonium acetate and acetonitrile gradient specified by the manufacturer was used for elution.

Western Blotting

Western blotting was performed and analyzed as previously described (Mexal et al. 2005). The blotted membranes were blocked for 1 h in 5% powdered milk diluted with Tris-buffered saline with 0.5% Tween-20 at room temperature and overnight at 4°C with the appropriate primary

antibody: goat anti-GAPDH (SC-20357; Santa Cruz Biotechnology), 1:1,000 dilution and goat anti-α7* (SC-1447, Santa Cruz Biotechnology), 1:1,000 dilution. The specificity of this antibody was first assessed using human skin fibroblasts, which do not express detectable levels of α7* receptors. Consistent with previous findings, we did not detect any antibody reactivity in lanes loaded with fibroblast protein homogenate (data not shown). Expression levels for α7* were normalized to GAPDH levels.

Statistical Analysis

All statistical analyses were performed using SPSS (two-way ANOVA; SPSS version 9, SPSS, Chicago, IL). QRT-PCR and western blot data were imported into Excel. Relative expression values were $\log_{10}$ transformed and a two-way analysis of variance was utilized as the primary statistical tool to detect significant changes in gene expression due to the main effect of schizophrenia or to the interaction of schizophrenia and smoking. Post hoc comparisons were conducted using Tukey's statistical analysis. Differences in mean gene expression were determined using a two-tailed Student's *t* test ($p<0.05$, Excel). Mean relative expression levels (±SEM) for each experimental group were graphed in Sigma Plot 2000 (SPSS). The distribution of *CHRNA7* genotypes across smoking and diagnostic groups was evaluated by a Fisher's exact test.

Continuous variables, including age, brain pH, freezer storage time, and postmortem interval (PMI), were evaluated by two-way ANOVA to determine if there were any significant differences across the four subject groups for our expression studies. These variables were also correlated with gene expression.

To determine whether gender or agonal state was significantly different across the mental illness and control groups, a Fisher's exact test was performed. The effect of gender, a categorical variable, was also evaluated by two-way ANOVA, smoking × gender effects, or three-way ANOVA, for smoking × schizophrenia × gender. To assess whether agonal state (Li et al. 2004; Mexal et al. 2006) affected mRNA or protein levels of the CHRNA7 gene, the expression for subjects who suffered fast deaths (agonal scores of 1 or 2) was compared to those who experienced intermediate to slow deaths (agonal scores of 3 or 4) by a Student's *t* test (Excel).

Other potential confounds, including alcohol use and antipsychotic drug use, were investigated. The effects of alcohol on gene expression were assessed by reanalyzing the data using a Student's *t* test (Excel) after excluding subjects who had used alcohol at the time of death. Antipsychotic drug use was investigated in the QRT-PCR analysis of the interactive effects of smoking and schizophrenia, which included three drug-naïve patients. Gene expression was compared by ANOVA between these unmedicated subjects and patients treated with either typical neuroleptics ($n=11$) or those who were on atypicals ($n=4$).

Results

Demographics

This study characterized the hippocampal gene expression profile for the α7 nicotinic receptor gene, *CHRNA7*, in control nonsmokers, control smokers, schizophrenic non-smokers, and schizophrenic smokers. Hippocampus from a total of 17 schizophrenics and 17 controls was analyzed by QRT-PCR (Table 1). In the western blot studies, 35 subjects were studied, 15 of which were included in the QRT-PCR experiments.

Gene Expression Analysis of the Interactive Effects of Schizophrenia × Smoking on CHRNA7 mRNA Expression

Several candidate genes displayed reduced expression in schizophrenic nonsmokers compared to all controls and schizophrenic smokers in our earlier microarray study (Mexal et al. 2005). These genes included the α7 nicotinic acetylcholine receptor subunit (*CHRNA7*). The *CHRNA7* gene is partially duplicated on the same chromosome (15q14) (Gault et al. 1998; Riley et al. 2002). Exons 5–10 of the full-length gene were duplicated in a large duplicon, which interrupted a partial duplication of a second gene. The chimeric gene, *CHRFAM7A*, is expressed as mRNA, and the α7 containing sequences are 99.9% homologous to the full-length *CHRNA7* gene (Gault et al. 1998; Riley et al. 2002). The function of the duplicated gene is not known. QRT-PCR was performed to differentiate the expression of the two highly homologous transcripts, *CHRNA7* and *CHRFAM7A*. Schizophrenic nonsmokers displayed reduced expression levels of the full-length *CHRNA7* gene relative to control nonsmokers and schizophrenic smokers in the microarray analysis (Fig. 1). The probes on the Applied Biosystem's gene chip were localized in the 3′ untranslated region of the gene. This region is duplicated in *CHRFAM7A*; the probes, thus, detect both the full-length *CHRNA7* gene and *CHRFAM7A*. Primer sets were designed to specifically amplify either *CHRNA7* or *CHRFAM7A* (Fig. 2a). Exponential amplification of *CHRNA7* was detected 10 cycles prior to the amplification of *CHRFAM7A* (data not shown), suggesting that the latter transcript is expressed at much lower levels than the full-length transcript in the human hippocampus. *CHRFAM7A* expression was not evaluated across groups; QRT-PCR conditions could not be optimized due to the limited detection of this transcript in postmortem human brain. However, consistent with the microarray findings, QRT-PCR with the specific primer sets detected significantly lower *CHRNA7* levels in schizophrenic nonsmokers compared to control nonsmokers and schizophrenic smokers (Fig. 2).

A similar gene expression pattern was also observed at the protein level (Fig. 2b). The reported *CHRNA7* expression by QRT-PCR and western blot analyses was not correlated with continuous variables such as age, pH, PMI, and storage time ($p>0.05$), and categorical parameters such as gender, agonal state, and alcohol use did not contribute to the expression changes (three-way ANOVA, $p>0.05$). Moreover, similar *CHRNA7* mRNA expression levels ($p>0.05$) were detected by QRT-PCR between the three drug-naïve patients, nine patients on typical antipsychotics, and four on atypicals, suggesting that this transcript was not altered by medication history ($p>0.05$).

Genetic Variation at *CHRNA7* and mRNA Expression

Single nucleotide polymorphisms within the 231-base pair core promoter of the *CHRNA7* gene have previously been shown by a luciferase reporter gene assay to significantly alter promoter activity in vitro, suggesting that transcription may be altered in individuals with these mutations (Leonard et al. 2002). Subjects evaluated in the current expression analysis were, therefore, screened for these polymorphisms, and possible correlations between genotype and *CHRNA7* mRNA expression were investigated. Overall, three proximal promoter polymorphisms were identified in our sample set, including −86 C/T, −194 G/C, and −46 G/T (numbering with respect to exon 1), all of which have been associated with significantly reduced luciferase expression in a reporter gene assay (Leonard et al. 2002). Schizophrenic nonsmokers had a greater proportion of promoter polymorphisms than control nonsmokers and schizophrenic smokers (Table 3), the two cohorts which displayed significantly greater mRNA expression than the schizophrenic nonsmoker group (Fig. 2b). However, the difference did not reach statistical significance. To investigate a possible association of promoter mutations with schizophrenic nonsmokers, we utilized an expanded DNA dataset from the laboratory that included Caucasian subjects collected locally in the Schizophrenia Center. This sample included a total of 48 control nonsmokers, 18 control smokers, 44 schizophrenic nonsmokers,

and 146 schizophrenic smokers. The increased sample revealed a statistically significant difference in the genotypic distributions between these groups with schizophrenic nonsmokers having a greater number of promoter variants (Table 3; schizophrenic nonsmokers versus control nonsmokers, p=0.0276; schizophrenic nonsmoker versus schizophrenic smoker, p=0.0174, Fisher's exact test, one-tailed).

Discussion

The α7 nicotinic receptor gene, *CHRNA7*, has been associated with schizophrenia, the P50 deficit and smoking in schizophrenia (Leonard et al. 2001, 2007). Surface expression of the assembled, functional pentamer is decreased in multiple postmortem brain regions of schizophrenic subjects (Court et al. 1999; Freedman et al. 1995; Guan et al. 1999; Marutle et al. 2001). Surface expression of low affinity α7* receptors, as measured by [^{125}I]-α-bungarotoxin in rodents, is only upregulated at high levels of nicotine (Marks et al. 1986). In humans, we found that [^{125}I]-α-bungarotoxin binding was increased in postmortem hippocampus of smokers but the increase was not significant (Breese et al. 2000). Other investigators do not find a difference (Court et al. 1999). Thus, binding may only increase in brain of very heavy smokers. De Luca et al. (2004) recently reported that there is no significant difference in *CHRNA7* mRNA expression levels in dorsolateral prefrontal cortex between schizophrenics and controls. However, detailed smoking histories for subjects in this study were not available.

Our current data show that both *CHRNA7* mRNA and protein expression were reduced in schizophrenic non-smokers relative to controls. In schizophrenic smokers, *CHRNA7* mRNA and protein expression is normal. Promoter mutations in the *CHRNA7* proximal promoter have been associated with decreased transcription and are found more frequently in schizophrenic subjects and controls with sensory-processing deficits (Leonard et al. 2002). Schizophrenic nonsmokers, in whom low levels of *CHRNA7* mRNA were found, appear to have a significantly greater proportion of promoter polymorphism compared to the other groups evaluated in this study. Moreover, and consistent with our in vitro findings (Leonard et al. 2002), we observed reduced *CHRNA7* mRNA expression in subjects carrying a promoter polymorphism, regardless of diagnosis and/or smoking status, although the difference was not significant. While a larger sample size is needed to accurately determine the significance of this association, reduced *CHRNA7* mRNA expression in schizophrenic nonsmokers could be due to the greater proportion of subjects carrying a promoter polymorphism compared to control nonsmokers and schizophrenic smokers. We also examined the relationship of *CHRNA7* promoter polymorphism to nonsmoking status in a larger, genotyped set (Table 3). With this increased number of subjects, we found significantly more promoter mutations in schizophrenic nonsmokers than in either control nonsmokers or schizophrenic smokers. This is consistent with our result in postmortem hippocampus and suggests that promoter polymorphism may account for low levels of *CHRNA7* mRNA in schizophrenic nonsmokers.

Our results also indicate that adequate intracellular protein for α7* receptor expression is present in schizophrenic smokers. We conclude that proper assembly of the receptor in the endoplasmic reticulum or trafficking of the holoreceptor to the cell surface is aberrant in schizophrenic patients. Disruption of the normal endoplasmic reticulum (ER) assembly process and/or maturation into competent surface receptors may, thus, account for decreased α7* receptor surface expression. These processes are not independent; improper assembly of receptors results in ER retention, and hence, a failure to reach the cell surface as functional receptors (Nicke et al. 2004; Sallette et al. 2004). The α7 subunit peptide crosses the membrane four times and is assembled as a pentamer. The initial step of receptor assembly is complex and inefficient (Wanamaker et al. 2003), a process influenced by posttranslational modifications of nAChRs (Drisdel et al. 2004; Sallette et al. 2005; Wanamaker et al. 2003), as

well as interactions with the ubiquitin-proteosome pathway (Christianson and Green 2004; Ficklin et al. 2005; Wanamaker et al. 2003) and non-receptor accessory/chaperone proteins (Blount and Merlie 1991; Chang et al. 1997; Jeanclos et al. 2001; Keller and Taylor 1999; Ren et al. 2005; Williams et al. 2005). Clearly, further investigation of the cascade of events leading to α7* nAChR cell surface expression in schizophrenics compared to non-mentally ill subjects is warranted. Efforts have already been made in characterizing the processes leading to the membrane expression of NMDA receptors, another neurotransmitter receptor system implicated in schizophrenia. A recent study suggested that alterations in the glutamatergic system in schizophrenia may not be due to receptor expression changes but to the abnormal expression of interacting proteins that are responsible for the trafficking of these receptors from the endoplasmic reticulum to the cell surface (Kristiansen and Meador-Woodruff 2005). It is, therefore, possible that many disease susceptibility genes, including the *CHRNA7*, may be affected at common levels of regulation that have yet to be identified.

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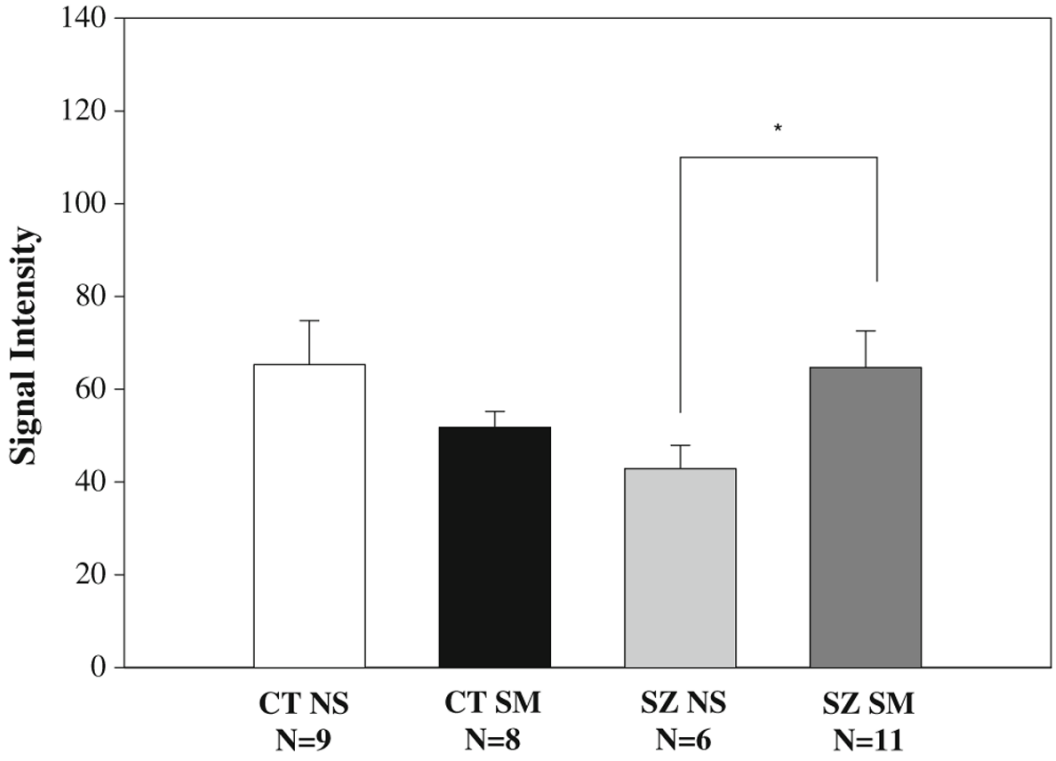

Figure 1.

CHRNA7 signal intensity is significantly reduced in schizophrenic nonsmokers compared to schizophrenic smokers. The probe set for *CHRNA7* on the oligonucleotide array detected significantly reduced signal intensity in schizophrenic nonsmokers compared to patient smokers. *$p<0.05$

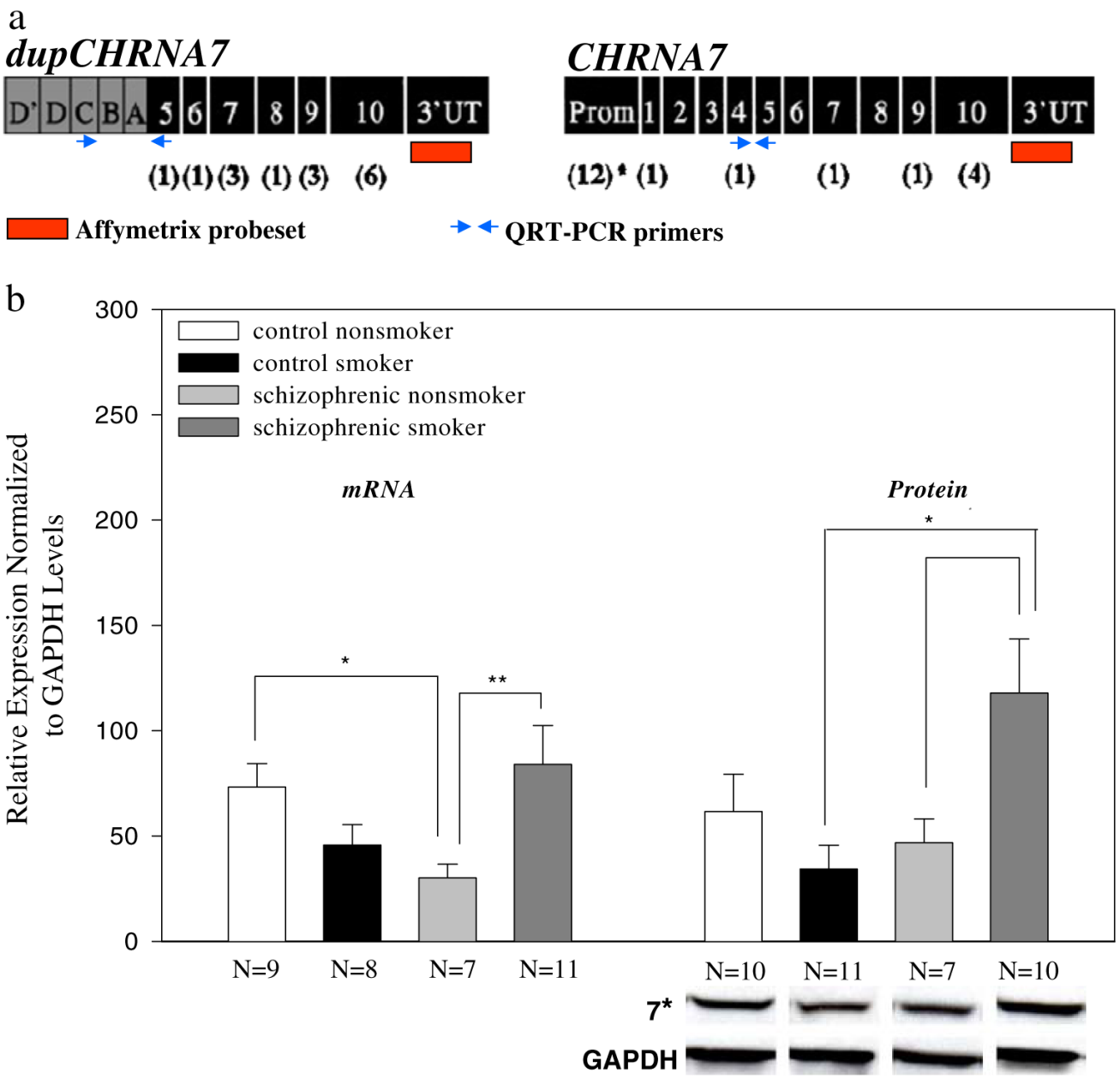

Figure 2.

CHRNA7 displays reduced mRNA and protein levels in schizophrenic nonsmokers. **a** The microarray probe set for *CHRNA7*, shown in *red*, was designed to detect expression from both the full-length *CHRNA7* and the duplicated fusion gene, *CHRFAM7A*. **b** Reduced α7 nAChR mRNA expression was observed in schizophrenic nonsmokers relative to smokers by RT-PCR and western blot analyses. QRT-PCR primers, shown in blue (**a**), were designed to specifically amplify either *CHRFAM7A* or *CHRNA7*. Schizophrenic nonsmokers displayed significantly reduced mRNA and protein expression relative to patient smokers. Patient nonsmokers also had lower expression compared to control nonsmokers, but this difference was statistically significant only at the mRNA level. Representative protein bands from western blots, below the graph, show the quantified protein amounts. QRT-PCR and western blot assays overlapped by 14 subjects (three control nonsmokers, three control smokers, four schizophrenic non-

smokers, and four schizophrenic smokers). Mean relative mRNA amounts (± SEM) for each subject group are shown. *$p<0.05$, **$p<0.01$

Table 1

Summary of demographics on postmortem tissue utilized for microarray, QRT-PCR, and western blot assays

Cohort	Age[a]	Gender		Race			Tobacco use (PPD)	Alcohol Use		Medication				Agonal Score		PMI[b] (hrs)	Brain pH	Tissue storage (months) [c]	Hem (L/R)
		M	F	AA	C	H		Yes	No	None	Typ	Atyp	Both	1 or 2	3 or 4				
Array																			
CT NS (*n*=9)	34.0±3.7	6	3	1	7	1	N/A	0	9	6	0	0	0	7	2	**12.3±2.6**[b]	6.4±0.1	**9.4±2.9**[c]	3/4
CT SM (*n*=8)	**30.6±4.3**[a]	5	3	0	8	0	1.1±0.24	3	5	6	0	0	0	6	2	**9.8±2.7**[b]	6.5±0.1	**35.8±12.3**[c]	7/0
SZ NS (*n*=6)	38.1±7.0	4	2	0	6	0	N/A	1	5	2	3	1	0	5	1	**16.8±4.0**	6.5±0.1	24.3±8.3	1/2
SZ SM (*n*=11)	**46.5±3.9**[a]	8	3	1	10	0	1.6±0.28	1	10	0	6	3	1	11	0	**16.9±2.2**	6.6±0.1	**16.2±2.3**[c]	9/1
QRT-PCR SMxSZ																			
CT NS (*n*=9)	35.5±4.4	6	3	1	7	1	N/A	0	9	6	0	0	0	7	2	**9.4±1.9**[b]	6.4±0.1	**13.3±3.7**[c]	3/4
CT SM (*n*=8)	**38.6±3.8**[a]	5	3	0	8	0	1.1±0.24	3	5	6	0	0	0	6	2	**12.7±3.0**[b]	6.5±0.1	**32.3±11.4**[c]	7/0
SZ NS (*n*=7)	**42.0±7.1**[a]	5	2	0	7	0	N/A	1	6	3	3	1	0	5	2	**16.8±3.3**	6.5±0.1	24.3±7.0	1/3
SZ SM (*n*=11)	**46.5±4.3**[a]	8	3	1	10	0	1.6±0.28	1	10	0	6	3	1	11	0	**14.3±1.4**	6.6±0.1	**16.1±2.6**[c]	9/1
Western																			
CT NS (*n*=9)	33.9±5.1	6	3	0	7	2	N/A	1	8	10	0	0	0	6	3	**13.4±2.2**[b]	6.5±0.1	69.0±16.3	4/2
CT SM (*n*=9)	32.8±2.9	6	3	1	8	0	1.5±0.2	2	7	11	0	0	0	7	2	**11.9±2.0**[b]	6.4±0.1	59.0±15.2	8/1
SZ NS (*n*=7)	36.6±4.3	5	2	2	5	0	N/A	2	5	2	3	1	1	7	0	**17.9±2.8**[b]	6.4±0.1	69.4±19.8	1/4
SZ SM (*n*=10)	35.0±3.1	7	3	0	8	2	1.8±0.3	1	9	0	6	3	1	10	0	**17.1±2.0**[b]	6.4±0.1	68.5±14.4	6/2

ll subjects evaluated by microarray analysis were included in QRT-PCR studies and 15 were included in the western blot analysis. Hemisphere information was available for 25/34 array subjects, 25/35 QRT-CR SM x SZ subjects, and 28/35 western subjects.

Age was significantly lower in schizophrenic relative to control smokers in the array study ($p<0.05$), and in schizophrenic smokers compared to control smokers and schizophrenic nonsmokers in the QRT-CR study ($p<0.05$).

PMI was significantly lower in all controls relative to schizophrenics in all three assays ($p<0.05$).

Tissue storage time was significantly higher in control smokers relative to control nonsmokers and schizophrenic smokers in both the array and QRT-PCR studies ($p<0.05$). AA, African American; C, Caucasian; I, Hispanic; F, female; M, male; NS, nonsmoker; SM, smoker; CT, control; SZ, schizophrenic; PPD, packs per day of cigarettes; Hem, hemisphere; L, left; R, right; atyp, atypical antipsychotic; typ, typical ntipsychotic; N/A, not applicable

Table 2

Sequence for primers utilized in SYBR green QRT-PCR and sequencing

Gene	Forward primer	Reverse primer	Product Size (bp)
QRT-PCR Primers			
CHRNA7	TTTACAGTGGAATGTGTCAGAATATCC	TGTGGAATGTGGCGTCAAG	125
CHRFAM7A	GGAATCCCAGATGAGAGAGCTTATC	TGTGGAATGTGGCGTCAAAG	236
GAPDH	GGTATCGGAAGGACTC	GGATGATGTTCTGGAGAGC	117
CHRNA7 proximal promoter	AGTACCTCCCGCTCACACCTCG	ATGTTGAGTCCCGGAGCTGCAG	271

Table 3

CHRNA7 proximal promoter genotypes across diagnostic and smoking groups

Diagnosis and smoking status	Genotype		
	***N/N* (%)**	**SNP (%)**	**Total**
Postmortem brains evaluated by QRT-PCR			
Control nonsmokers	7 (77.8)	2 (22.2)	9
Control smokers	6 (75.0)	2 (25.0)	8
Schizophrenic nonsmokers	4 (57.1)	3 (42.9)	7
Schizophrenic smokers	7 (63.6)	4 (36.4)	11
Total	24	11	35
Expanded dataset			
Control nonsmokers[a]	40 (83.3)[a]	8 (16.7)	48
Control smokers	14 (77.8)	4 (22.2)	18
Schizophrenic nonsmokers[a,b]	28 (63.6)[a,b]	16 (36.4)	44
Schizophrenic smokers[b]	118 (80.8)[b]	28 (19.2)	146
Total	200	56	256

SNP single nucleotide polymorphism

[a] Schizophrenic nonsmokers versus control nonsmokers (p=0.0276, Fisher's exact test, one-tailed)

[b] Schizophrenic nonsmokers versus schizophrenic smokers (p= 0.0174, Fisher's exact test, one-tailed)