Molecular Psychiatry (2004) 9, 299–307

www.nature.com/mp

ORIGINAL RESEARCH ARTICLE

Expression analysis of neuregulin-1 in the dorsolateral prefrontal cortex in schizophrenia

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Genetic linkage and association have implicated neuregulin-1 (NRG-1) as a schizophrenia susceptibility gene. We measured mRNA expression levels of the three major isoforms of NRG-1 (ie type I, type II, and type III) in the postmortem dorsolateral prefrontal cortex (DLPFC) from matched patients and controls using real-time quantitative RT-PCR. Expression levels of three internal controls—GAPDH, cyclophilin, and β-actin—were unchanged in schizophrenia, and there were no changes in the absolute levels of the NRG-1 isoforms. However, type I expression normalized by GAPDH levels was significantly increased in schizophrenia DLPFC (by 23%) and positively correlated with antipsychotic medication dosage. Type II/type I and type II/type III ratios were significantly decreased (18 and 23% respectively). There was no effect on the NRG-1 mRNA levels of genotype at two SNPs previously associated with schizophrenia, suggesting that these alleles are not functionally responsible for abnormal NRG-1 expression patterns in patients. Subtle abnormalities in the expression patterns of NRG-1 mRNA isoforms in DLPFC may be associated with schizophrenia.
Molecular Psychiatry (2004) **9,** 299–307. doi:10.1038/sj.mp.4001434
Published online 21 October 2003

Keywords: neuregulin-1; schizophrenia; expression; postmortem brain; isoform; dorsolateral prefrontal cortex

Introduction

Schizophrenia is a complex genetic disorder affecting 0.5–1% of the general population worldwide. Several genome-wide linkage scan studies and meta-analysis of whole-genome linkage scans show a suggestive linkage to schizophrenia on chromosome 8p.[1–10] Recently, neuregulin-1 (NRG-1), which maps to the 8p locus, has been implicated as a susceptibility gene for schizophrenia by a combination of linkage and association analyses.[11,12] NRG-1 is one of the neuregulin family of proteins, which have a broad range of bioactivities in the central nervous system and contain an epidermal growth factor (EGF)-like motif that activates membrane-associated tyrosine kinases related to ErbB receptors.[13] The EGF-like domain of NRG-1 is required for ErbB receptor binding, dimerization, tyrosine phosphorylation, and activation of downstream signaling pathways.[14] A gene-targeting approach for NRG-1-ErbB signaling revealed a behavioral phenotype in mice that overlaps with certain animal models for schizophrenia. For example, NRG-1 and ErbB4 mutant mice exhibit elevated activity levels in an open field, which was reversed by clozapine, and abnormal sensorimotor gating measured by prepulse inhibition of the startle reflex.[11,15]

The NRG-1 gene generates multiple alternative splicing variants, classified into three primary isoform groups.[16] NRG-1 type I (heregulin/ARIA: acetylcholine receptor-inducing activity/NDF: neu differentiation factor) has an immunogloblin-like domain, followed by a region of high glycosylation; type II (GGF: glial growth factor) has GGF-specific and immunogloblin-like domains; and type III (SMDF: sensory and motor neuron-derived factor) has a cysteine-rich domain. These NRG-1 isoforms play multiple and distinct functions in neuronal development, and abnormalities in brain development have been implicated in schizophrenia. Moreover, NRG-1 regulates the expression and plasticity of *N*-methyl-*d*-aspartate receptors (NMDAR), of the β2 subunit of the γ-amino butyric acid receptor, and of nicotinic acetylcholine receptor subtypes including α5, α7, and β4 subunits[17–20], some of which also may be involved in genetic risk for schizophrenia.[21,22]

Thus, while genetic evidence implicates NRG-1 as a schizophrenia susceptibility gene, and the biology of NRG-1 overlaps with diverse aspects of the putative biology of schizophrenia, there have been no published studies of NRG-1 expression in the schizophrenic brain tissue, and little is known about whether a specific NRG-1 isoform contributes to the risk for schizophrenia. Here, we employed a real-time quantitative RT-PCR technique to explore the mRNA

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Received 21 April 2003; revised 25 August 2003; accepted 10 September 2003

expression of each type of NRG-1 in the dorsolateral prefrontal cortex (DLPFC), where prominent functional and neuroanatomical abnormalities have often been observed in schizophrenia.[23]

Materials and methods

Human postmortem tissue and RNA extraction
Postmortem DLPFC tissues from brains were collected at the Clinical Brain Disorders Branch, as previously described. [24] Diagnoses were retrospectively established by two psychiatrists using DSM-IV criteria. We endeavored within practical limits to derive a rough approximation of lifetime neuroleptic exposure, recognizing that this is an uncertain estimate. All available records, including inpatient and outpatient clinic records, were meticulously reviewed for every subject. Each reference, anywhere in the chart, to a new medication and to a change in dose of an old medication was catalogued. While it was impossible to exclude potential discontinuities in treatment (or patient noncompliance), in general, contiguous dose information was available for almost every subject. The total daily dose of neuroleptic medication given to the patients was calculated by adding the various daily medication levels and converting these levels to chlorpromazine (CPZ) equivalents, as previously formulated.[25] A median value of drug dosage was then derived from the CPZ equivalents to give the estimated average daily dose; this value was multiplied by the duration of illness (estimated from the earliest age of definable symptoms or age at first hospitalization) to give the estimated lifetime CPZ equivalents. Samples were matched for age, gender, ethnicity, brain pH, hemisphere, postmortem interval (PMI), and months in freezer (MIF). Demographic data are shown in Table 1.

The tissue blocks were dissected from the middle, superior, or inferior frontal gyrus from a 1–1.5 cm coronal slab just anterior to the corpus collosum. The blocks contained primarily gray matter and a small, but presumably random, amount of white matter. In order to test for the possibility of systematic difference in the gray matter/white matter ratio in the dissections of PFC from patients and controls, the total RNA extracted from these blocks was screened by microarray expression profiling for the content of mRNAs highly expressed in white matter such as glial fibrillary acidic protein (GFAP) and myelin basic protein (MBP). No significant differences in GFAP mRNA levels or MBP mRNA levels in RNA from patients with schizophrenia compared to controls were found (M Vawter, personal communication). While this approach does not conclusively rule out a systematic difference in the ratio of gray to white matter compartments in the tissue sampled from the schizophrenic and control groups, it reduces the likelihood of such an artifact.

The tissues were pulverized and stored at −80°C until use. Total RNA was extracted from 300–500 mg of DLPFC using TRIZOL Reagent (Life Technologies Inc., Grand Island, NY, USA), as previously described.[26] The yield of total RNA was determined by absorbance at 260 nm and the quality of total RNA was also analyzed using agarose gel electrophoresis.

DNAse treatment and reverse transcriptase reaction
Total RNA was treated with DNase for the removal of contaminating genomic DNA using DNase Treatment & Removal Reagents (Ambion, Austin, TX, USA), according to the manufacturer's protocol. After DNase treatment, the quality of total RNA was examined using agarose gel electrophoresis. Total RNA (6.8 μg) treated with DNase was used in 50 μl of reverse transcriptase reaction to synthesize cDNA, by using a SuperScript first-strand synthesis system for RT-PCR (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. Briefly, total RNA (6.8 μg) was denatured with 1 mM of dNTP and 5 ng/μl of random hexamers at 65°C for 5 min. After the addition of RT buffer, $MgCl_2$ (5 mM in final concentration), dithiothreitol (10 mM in final concentration), RNAseOUT recombinant ribonuclease inhibitor (100 U), and SuperScriptII RT (125 U), the reaction mixture was incubated at 25°C for 10 min, at 42°C for 40 min, and at 70°C for 15 min. RNAse H (5 U) was added to the reaction mixture and then incubated at 37°C for 20 min.

Real-time quantitative PCR
The structure of human NRG-1 transcripts annotated in NCBI databases and the locations of each PCR amplicon are shown in Figure 1. We designed specific primer and probe combinations to recognize each NRG-1 isoform family as follows: type I: exons 4 and 5, type II: exons 4 and 8, and type III: exons 7 and 8.

NRG-1 mRNA expression levels were measured by real-time quantitative RT-PCR, using each combination of oligonucleotides and an ABI Prism 7900 sequence detection system with 384-well format (Applied Biosystems, Foster City, CA, USA). Each 20 μl PCR reaction contained 6 μl of cDNA, 900 nM of each primer, 250 nM of probe, and 10 μl of TaqMan Universal PCR Mastermix (Applied Biosystems) containing AmpliTaq Gold DNA polymerase, AmpErase UNG, dNTPs with dUTP, passive reference, and optimized buffer components. The PCR cycling conditions were 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s, and 59°C or 60°C for 1 min. PCR data were obtained with the Sequence Detector Software (SDS version 2.0, Applied Biosystems) and quantified by a standard curve method. This software plotted the real-time fluorescence intensity and selected the threshold within the linear phase of the amplicon profile. The software plotted a standard curve of the cycle at threshold (Ct) (where the fluorescence generated within a reaction and threshold crosses) *vs* quantity of RNA. All samples were measured in one plate for one target gene or isoform, and their Ct values were in the linear range of the standard curve. Experiments were typically performed three times with triplicate determination and each gene expression level was determined by the

Table 1 Demography in the formation of the Clinical Brain Disorder Branch cohort

Case number	*Diagnosis*	*Age*	*Sex*	*Race*	*Side*	*pH*	*PMI (h)*	*Month in freezer*	*Cause of death*	*Manner of death*	*Age of onset/ duration of illness (year)*	*Last CPZ (eq/mg)*	*Daily CPZ (eq/mg)*	*Lifetime CPZ (eq/kg)*
1	CON	68	M	AA	L	6.57	22.5	192	ASCVD	natural				
2	CON	58	F	AA	L	6.54	26.5	226	ASCVD	natural				
3	CON	39	F	AA	L	6.34	40.5	226	Cardiac arrest	natural				
4	CON	46	F	AA	L	5.93	19.5	147	Dilated cardiomyopathy	natural				
5	CON	45	M	C	L	6.61	16.0	144	Blunt force injuries	accident				
6	CON	47	M	AA	L	6.03	60.0	157	Acute bronchial asthma	N/A				
7	CON	77	M	AA	R	6.06	18.5	146	Occlusive coronary atherosclerosis	natural				
8	CON	55	M	AA	R	6.00	9.5	146	MI (ASCVD)	natural				
9	CON	60	F	C	L	6.40	8.0	145	ASCVD	natural				
10	CON	61	F	AA	R	6.15	61.0	145	Multiple blunt force injuries	accident				
11	CON	26	M	C	L	6.08	13.0	115	ASCVD	natural				
12	CON	52	F	AA	R	6.87	10.0	100	Ruptured aorta	natural				
13	CON	42	M	AA	R	6.63	40.0	97	Acute asthma attack	natural				
14	CON	24	M	AA	R	6.59	12.5	96	Fibrinous pericarditis	natural				
15	CON	38	M	AA	R	6.14	32.5	95	Pulmonary embolism	accident				
16	CON	56	M	AA	R	6.09	33.0	88	Pulmonary embolism	natural				
17	CON	57	F	AA	R	6.43	19.0	76	MI-ASCVD	natural				
18	CON	59	F	AA	R	6.57	37.0	72	Cirrhosis of the liver	natural				
19	CON	67	F	AA	L	6.69	34.0	67	Cardiomyopathy Pulmonary edema	natural				
Mean (SD)		51.4 (13.8)				6.35 (0.28)	27.0 (15.8)	130.5 (47.8)						
20	SCH/TD	71	F	C	L	6.41	47.5	181	ASCVD	natural	15/56	100	500	0.6
21	SCH	36	M	AA	R	6.56	13.0	192	Blunt force injuries	suicide	21/16	400	850	5
22	SCH	46	M	AA	R	6.35	24.5	192	ASCVD	natural	23/23	N/A	N/A	N/A
23	SCH	44	F	AA	R	6.51	32.5	191	Cardiomegaly and hypertension	natural	19/15	200	200	1.1
24	SCH	46	M	AA	R	6.73	25.0	196	Blunt force injuries	suicide	36/10	300	300	1.1
25	SCH	48	M	C	R	6.29	13.5	146	Delusional hyponatremia and hypo-osmolar coma	undetermined	33/15	300	300	1.6
26	SCH	73	M	C	R	6.00	13.5	143	ASCVD	natural	23/50	450	450	4.6
27	SCH	34	M	AA	R	6.23	34.5	141	Acute benzotropine intoxication	undetermined	26/8	N/A	N/A	N/A
28	SCH	75	M	AA	L	6.29	41.5	121	Undetermined	natural	29/46	400	400	5.3
29	SCH	64	F	AA	R	6.48	19.5	121	Asphyxia due to aspiration	accident	19/45	900	400	5.3
30	SCH	67	F	AA	R	6.63	38.5	118	Chronic obstructive pulmonary disease	natural	30/37	80	100	1.3
31	SCH	31	M	C	R	6.46	14.0	112	Cerebral edema	natural	17/14	200	N/A	N/A
32	SCH	23	M	AA	L	6.48	42.5	112	Respiratory arrest	natural	21/2	400	480	0.033
33	SCH	60	F	AA	L	6.38	19.0	110	ASCVD	natural	40/20	100	100	0.7
34	SCH	30	M	AA	L	6.32	72.5	106	Pneumonia	natural	18/12	1900	500	2.2
35	SCH	81	F	C	R	6.78	11.0	100	ASCVD	natural	27/54	100	150	2.1

Table 1 Continued

Case number	*Diagnosis*	*Age*	*Sex*	*Race*	*Side*	*pH*	*PMI (h)*	*Month in freezer*	*Cause of death*	*Manner of death*	*Age of onset/ duration of illness (year)*	*Last CPZ (eq/mg)*	*Daily CPZ (eq/mg)*	*Lifetime CPZ (eq/kg)*
36	SCH/TD	61	F	AA	R	6.74	20.0	95	Asphyxiation	accident	24/27	N/A	200	2
37	SCH/TD	38	M	AA	R	6.50	61.0	89	Acute peritonities	accident	16/27	800	60	11.8
38	SCH	41	F	AA	R	6.08	51.0	80	ASCVD	natural	16/25	2400	1135	10.4
39	SCH/TD	41	M	AA	L	6.63	32.0	76	ASCVD	natural	20/21	50	400	2.5
Mean		50.5				6.44	31.3	131.1			23.7/26.2	534	343	3.03
(SD)		(17.1)				(2.21)	(17.3)	(39.8)			(7.0/16.3)	(659)	(287)	(3.35)

Means and standard deviations are printed below the last individual in each group. CON=normal control; SCH=schizophrenia; TD=tardive dyskinesia; M=male; F=female; AA=African American; C=Caucasian; R=right; L=left; PMI=post-mortem interval; N/A=not available; CPZ=chlorpromazine; eq=equivalent.

Figure 1 NRG-1 structure and probe design. Human NRG-1 mRNA has 15 exons. The exon usages of type I, type II or type III isoforms of NRG-1 are shown. Arrows indicate the location of primers and probes specific for type I, type II or type III isoforms of NRG-1.

average of three independent experiments. Predicted Ct values and sample quantities were used for statistical analysis.

Oligonucleotide primers and standard curve construction

Primer and probe sequences were designed by using PRIMER EXPRESS software (version 2.0, Applied Biosystems). Agarose gel electrophoresis was used to verify the size predictions of PCR amplicons (data not shown). The TaqMan Pre-Developed Assay Reagent kit (Applied Biosystems) was used for housekeeping genes: GAPDH, β-actin, and cyclophilin. The real-time PCR (TaqMan) detection of NRG-1 isoforms used the following oligonucleotides: type I, forward primer P3089 5′-GCCAATATCACCATCGTGGAA-3′, reverse primer P3090 5′-CCTTCAGTTGAGGCTGGCATA-3′, probe P3091 5′-FAM-CAAACGAGATCATCACTG-MGB-3′; type II, forward primer P3092 5′-GAATCA-AACGCTACATCTACATCCA-3′, reverse primer P3093 5′-CCTTCTCCGCACATTTTACAAGA-3′, probe P3094 5′-FAM-CACTGGGACAAGCC-MGB-3′; type III, forward primer P3095 5′-CAGCCACAAACAACAGAA-ACTAATC-3′, reverse primer P3096 5′-CCCAGTG-GTGGATGTAGATGTAGA-3′, probe P3097 5′-FAM-CCAAACTGCTCCTAAAC-MGB-3′(purchased from Applied Biosystems). These primers were designed to amplify specific transcripts based on the unique exon structure of each isoform. Thus, for example, because isoform II lacks exons 5–7, primers focused on exons 4 and 8, which are contiguous in the isoform II transcript, and will amplify only this isoform. Standard curves for the housekeeping genes and the three NRG-1 isoforms were prepared using serial dilutions (1 : 4) of pooled cDNA from total RNA derived from DLPFC of six normal control subjects (Figure 2). In each experiment, the R^2 value of the standard curve was more than 0.99 and no-template control assays resulted in no detectable signal.

SNP genotyping

DNA was extracted from brain tissue using standard methods. P3149SNP8NRG221533 and SNP8NRG24-

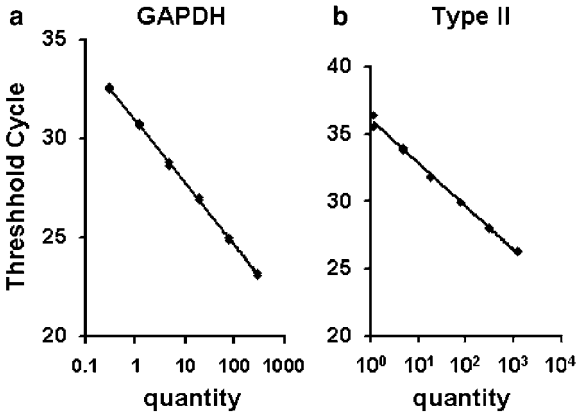

Figure 2 Standard curves for control (housekeeping genes) and NRG-1 type II. Standard curves for GAPDH (a) and type II (b). The quantity represents an amount of cDNA prepared from 1 ng of total RNA in the PCR reaction. R^2 values are 1.000 and 0.995 for GAPDH and type II.

3177P3155 genotypes were determined using the Taqman 5′-exonuclease allelic discrimination assay. These SNPs were chosen because they showed the strongest association to schizophrenia in prior studies.[11,12] Probes and primers for detection of the SNP are: SNP8NRG221533, forward primer P3151 5′-AAGGCATCAGTTTTCAATAGCTTTTT-3′, reverse primer P3152 5′-TAAGTAGAAATGGGAACTCTCCATCTC-3′, probe1 P3149 5′-FAM-TTTATTTTgCCAAATAT-MGB-3′, probe2 P3150 5′-VIC-TCTTTATTTTaCCAAATATCAT-MGB-3′; SNP8NRG243177, forward primer P3159 5′-AATTAGTAGGATTGGATGTTTGAACCA-3′, reverse primer P3160 5′-GATGGAGCGCTTCAGGAGAA-3′, probe1 P3155 5′-FAM-CCAGTATACgTTCACTTG-MGB-3′, probe2 P3156 5′-VIC-CCAGTATACaTTCAC-TTGA-MGB-3′. Each 10 μl PCR reaction contained 10 ng of DNA, 1 μM of each primer, 100 nM of each probe, and 5 μl of TaqMan Universal PCR Mastermix (Applied Biosystems) containing AmpliTaq Gold DNA polymerase, AmpErase UNG, dNTPs with dUTP, passive reference, and optimized buffer components. The PCR cycling conditions were at 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min.

Statistical analysis

An independent *t*-test was used to compare the age, brain pH, months in freezer, and postmortem interval, and a Mann–Whitney *U*-test was used to compare the gene expression levels between schizophrenic and control groups with Statistica software (release 5.5, Statsoft, Inc., Tulsa, OK, USA). The groups did not differ in gender and ethnicity. Differences in NRG-1 expression levels between groups were also analyzed by ANCOVA, with diagnosis as the independent factor and brain pH and age as covariates. Spearman rank order correlation test was used for comparison between demographic data and expression data.

Results

Control genes and NRG-1 mRNA levels

The expression levels of three standard 'housekeeping' genes—GAPDH, β-actin, and cyclophilin—were not significantly different between groups (Figure 3a). Raw (ie nonnormalized) NRG-1 isoform expression levels also did not differ between groups (Figure 3b).

Effects of demographics on NRG-1 mRNA expression levels

Expression levels of all the three NRG-1 isoforms normalized by cyclophilin were positively correlated with age in normal control subjects (Rho=0.637, P=0.006; Rho=0.573, P=0.015, and Rho=0.637, P=0.013 for type I, type II, and type III, respectively); however, there was no correlation between normalized NRG-1 isoform expression levels and age in schizophrenia patients (all $P>0.6$). Similar results were obtained with normalization to GAPDH and β-actin (data not shown). NRG-1 expression levels were not associated with sex, race or hemisphere, and did not correlate with PMI or MIF. Brain pH and type I mRNA expression normalized by cyclophilin were correlated in both normal control (Rho=−0.477, P=0.050) and schizophrenia groups (Rho=−0.500,

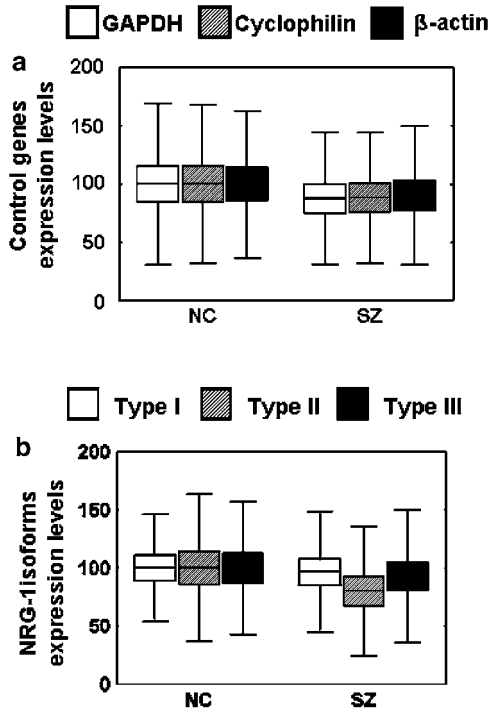

Figure 3 mRNA expression levels of NRG-1 isoforms. The expression levels of housekeeping genes (a) and NRG-1 isoforms (b) were measured in the DLPFC of normal control subjects (NC) and patients with schizophrenia (SZ). The expression levels were calculated by comparison to the percentage of average of normal control subjects. Boxes and bars outside boxes represent the standard error and standard deviation. Bars in boxes represent means.

P=0.041), with similar results normalizing by GAPDH or β-actin. Thus, brain pH (as well as age) was used as covariate for type I expression data analysis.

Normalized NRG-1 mRNA levels

NRG-1 type I expression levels normalized by GAPDH, cyclophilin or β-actin (to reduce the effects of possible mRNA degradation not detectable by electrophoresis and/or possible variations in RT efficiency) were increased by 23, 18 or 16%, respectively, in schizophrenia patients (ANCOVA: all $P<0.050$). (Figure 4a). No significant differences were observed between groups in normalized NRG-1 type II and type III expression levels (Figure 4b, c).

We further analyzed the expression ratios among the three types of NRG-1 isoforms to investigate possible isoform–isoform interactions or altered regulation of splicing (Figure 5). There was no significant difference in type I/type III expression ratio with or without covariates (age and pH) (normal control: 100.0 (mean) $\pm$33.8 (SD) *vs* schizophrenia patients: 109.1$\pm$36.8). However, both type II/type III and type II/type I expression ratios were significantly decreased in the schizophrenic group (17 and 23%, Mann–Whitney U-test: P=0.010 and 0.013, respectively). ANCOVA with brain pH and age as covariates did not alter the statistical significance of this relative type II decrease (type II/type I; F=10.96, P=0.002, df=1, 32).

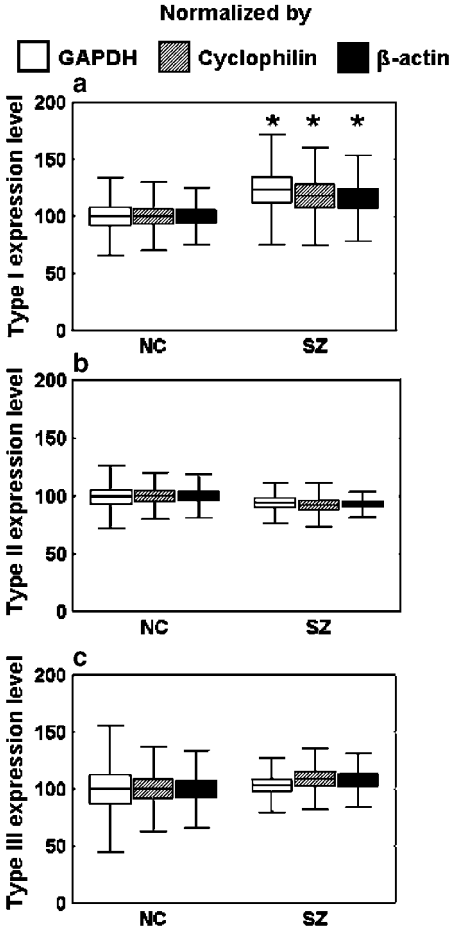

Figure 4 Relative expression levels of NRG-1 type I (a), type II (b), and type III (c) isoforms normalized by GAPDH, cyclophilin or β-actin in the DLPFC of normal control subjects (NC) and patients with schizophrenia (SZ). Significant group differences by ANCOVA are indicated by $^*P<0.05$.

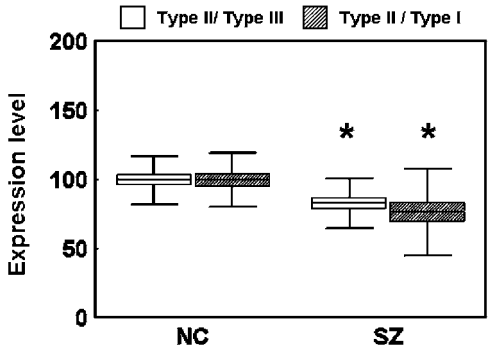

Figure 5 Relative expression ratios of Type II normalized by Type I or Type III in DLPFC of normal control subjects (NC) and patients with schizophrenia (SZ). $^*P < 0.05$.

Influence of clinical characteristics on NRG-1 expression

None of the measurements of NRG-1 isoforms correlated significantly with the age of onset, duration of illness or last and lifetime dose of chlorpromazine equivalents (data not shown). A positive correlation between type I expression levels normalized by cyclophilin and daily dose was found (Rho=0.601, P=0.014), although daily dose was not correlated with normalized type II or type III expression levels (Rho=−0.315, P=0.218; Rho=−0.102, P=0.681, respectively). Similar results were obtained after normalization by GAPDH and β-actin (data not shown).

Allele effects on NRG-1 expression

No effect of SNP8NRG221533 genotype, which has been reported to be associated with schizophrenia in both Icelandic and Scottish populations,[11,12] was apparent in type I, type II, and type III expression levels normalized by cyclophilin, and the expression ratio of type II/type III in total subjects, normal controls or patients. For example, one allele homozygote (N=21) had mean levels of type I expression of 100.9$\pm$37.6 (SD), while two carriers (N=12) had 99.2$\pm$28.3 (SD) ($P>0.8$). Neither NRG-1 expression levels normalized by GAPDH or β-actin nor the other combinations of isoform–isoform expression ratio were affected by this genotype in any group (data not shown). Similar negative results were obtained between NRG-1 expression and SNP8NRG243177 (data not shown), which also has been associated with schizophrenia.[11,12]

Discussion

In this study, we have measured mRNA expression levels of NRG-1 isoforms in DLPFC using real-time quantitative RT-PCR in patients with schizophrenia and in normal controls. NRG-1 has been implicated as a susceptibility gene in schizophrenia. We found preliminary evidence that the pattern of expression of NRG-1 isoforms may be abnormal in schizophrenia. Specifically, there was a small increase in type I expression levels, and a small decrease of type II/type I and type II/type III ratios in the patients with schizophrenia. As consistent results were obtained from normalization of NRG-1 isoforms by all the three housekeeping genes, our findings would seem to be robust at least in comparison to results that might have been based on using only one control gene. Our data appear to add to the evidence that NRG-1 may be involved in schizophrenia, but other explanations, for example, differences in postmortem stability of the various isoforms, cannot be excluded. Moreover, as our study did not include measurement of the levels of NRG-1 proteins, of expression in other brain regions or in other psychiatric disorders, further work is necessary to clarify whether changes in NRG-1 mRNA impact on protein expression and is regionally and diagnostically specific.

NRG-1 binds to its receptor, ErbB, and NRG-1-ErbB signaling plays multiple roles in development and plasticity in the central nervous system.[13] Type I is prominently expressed early in development; type II is abundantly expressed in the adult nervous system; and type III is the major isoform produced by sensory neurons and motorneurons, and is also expressed in the rodent brain.[27] Little is known about NRG-1 expression in human brain; however, NRG-1 is present in neuronal cell bodies and synapse-rich regions in the hippocampus and type II isoform is expressed in oligodendrocytes, astrocytes, and microglia.[28,29] We detected mRNA of each of the three major classes of NRG-1 isoforms in human DLPFC, but we did not characterize the multiple spice variants within these isoforms. We also found a positive correlation between expression levels of each of the NRG-1 isoforms with age in normal subjects, suggesting that NRG-1 mRNA increases as the prefrontal cortex ages. However, the meaning of this correlation is unclear, and it was not found in the patients.

NRG-1 type I has been implicated in neuronal plasticity because it shows activity-dependent regulation, and it is involved in regulating neurotransmitter receptor expression. Multiple perturbations in neuronal activity have been shown to affect type I expression. For example, seizures, long-term potentiation, and forced locomotion induce type I expression in the hippocampus, amygdala, and motor cortex. Brain injury induces NRG-1 protein expression in astrocytes of rat cerebral cortex.[30] Curare blockade of nicotinic receptors reduces the expression of type 1 protein in chick motor neurons, an effect that can be prevented by brain-derived neurotrophic factor and neurotrophin 3.[31] In the central nervous system, NRG-1 promotes the switch from the immature form of NMDAR, which contains primarily NR2B subunits to one containing more NR2C subunits.[17] NRG-1 also potentiates $\alpha 7$ nicotinic acetylcholine receptor transmission in hippocampal neurons,[20] and expression of the $\beta 2$ subunit of the γ-amino butyric acid receptor in cerebellar granule cells.[19] Thus, the relative increase in type I expression in schizophrenia brain might alter neuronal signaling of NRG-1 *per se*, or it may be an indirect factor in putative abnormalities of NMDA, nicotinic, and/or GABA receptor-related signaling in schizophrenia brain.[21,32,33] The positive correlation between type I expression level and the daily dose of chlorpromazine equivalents suggests that this upregulation of type I could reflect a relationship between NRG-1 expression level and illness severity. Alternatively, it might be due to neuroleptic treatment. We are currently exploring in animals the potential effect of antipsychotic medication on NRG-1 expression.

NRG-1 type II (GGF) is also of central importance for neuronal and glial development. Type II is expressed in developing cortical neurons, and it promotes the transformation and differentiation of radial glial cells, which in turn support cortical neuronal cell migration and differentiation.[34] A study using NRG-1-deficient mice revealed that NRG-1-erbB2 signaling is required for the establishment of radial glia and their transformation into astrocytes in cerebral cortex.[35] In our study, decreased ratios of type II/type I and type II/type III may be due to relative underexpression of type II in DLPFC of schizophrenia patients. Neuroanatomical abnormalities have been reported in DLPFC in schizophrenia, including abnormal neuropil and cytoarchitecture.[36–40] It is unclear whether variations in NRG-1 expression could relate to these changes. In addition, a change in the balance of type I/type II to type III NRG-1 may influence cholinergic neurotransmission, as the distinct isoforms differentially induce various subunits of the nAChR.[18]

Although NRG-1 was first recognized to be critical for multiple stages of schwann cell development[41,42], its role in promoting the development of myelin-forming cells is now recognized to include oligodendrocytes. Not only is NRG-1 and various ErbB receptors expressed in the subependymal zone and the forebrain oligogenic zone, but NRG-1 can also induce the division[43] and/or promote the differentiation of oligodendrocyte precursors *in vitro*.[44–47] It is conceivable that relatively decreased type II mRNA expression may relate to putative abnormalities of oligodendroglial function implicated in schizophrenia.[48,49] Finally, we did not find evidence that NRG-1 type III mRNA expression levels are changed in schizophrenia DLPFC, although this isoform also has effects on neuronal plasticity and development.[18,50]

The multiple marker haplotype in the NRG-1 gene that has been associated with schizophrenia spans the

first exon, which is the promoter region for type II and is far upstream from the exons of all other isoforms.[11,12] The functional allele contributing to the increased risk for schizophrenia has not been identified in NRG-1, nor is there evidence that any of the associated variations would impact gene expression or function. As two single SNPs associated with schizophrenia were located around the promoter region of NRG-1 and the first exon of GGF, these SNPs might regulate the expression levels of NRG-1 isoforms and/or isoform–isoform ratios. However, no obvious allele effects of these SNPs on NRG-1 expression patterns were observed in this small sample. The estimated relative risks of each of these markers alone were less than that of the seven-marker core haplotype.[11,12] Taken together, these two SNPs do not appear to be functional alleles, at least in terms of the regulation of NRG-1 expression in human DLPFC. However, the possible relative decrease in type II expression may be regulated by an as yet unidentified allele in linkage disequilibrium with the associated haplotype.

Our findings offer preliminary evidence that abnormal expression of NRG-1 isoforms in DLPFC may be related to the pathophysiology of schizophrenia, but the evidence is weak. The biologic implications of our results are unknown, but they are at least conceptually consistent with evidence that schizophrenia involves genetic abnormalities in developmental/plasticity-related processes.[51,52] Additional studies are needed to characterize NRG-1 expression in schizophrenia, including slide-based mRNA analyses, protein analyses, neuroleptics effects, diagnostic specificity, and further exploration of genotype based variation.

Acknowledgements

We wish to thank Drs Takashi Morihara, Toyoko Hiroi, and Masasumi Kamohara for advice on real-time quantitative PCR. We also thank Dr Kenichiro Miura for advice of statistical analysis and Dr Vakkalanka Radhakrishna and Dr Bhaskar Kolachana for SNP genotyping and Dr Gerry Fischbach for review of the manuscript. This work was supported in part by the Essel Foundation through a NARSAD distinguished investigator award to DRW.

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