図3　TIPSで用いられている学生用教育パンフレット（文献2より引用）

コメント：統合失調症（父親）とうつ病（母親）の家族歴があり，筆者らが追跡していた個体が「大学受験，父親の統合失調症の再発，母親のうつ状態の悪化」というストレス条件下で幻聴，自生記憶想起を体験したが，治療に反応して速やかに症状が消褪した症例である。薬物療法を中止してから8年以上経過しているが，現在までのところ一貫して安定している。本論のテーマ「統合失調症の早期発見・発症予防の可能性」と重なる部分の大きい症例と思われるため，紹介させていただいた。なお本症例の診断は，一過性精神病性障害を考えている。

2. 予防への寄与を目指す疾患教育の試み

筆者らは，統合失調症の子弟（ハイリスク児）の追跡研究[8]や統合失調症患者の病前行動特徴の研究[9]から，統合失調症の1次・2次予防の実現には3つのポイントがあると考えている（表6）。

1つ目のポイントは，病前特徴への働きかけである。ハイリスク児研究や病前特徴研究の結果から，統合失調症患者は発症前から「消極的，自信がない，対人緊張が強い，非社交的で孤立しがち」などの性格・行動上の特徴を示す場合が多く，発症しない人は「自己肯定的で自己評価が安定，積極的で自主性がある，対人関係が円満」などの対照的な特徴を示す場合が多いことが明らかになった。発症者で乏しく発症しない人で認められやすい「対人関係能力，問題処理技能，自己評価」は，発症に防御的に働く抗罹病効果を持つ可能性がある。そこで予防のポイントの1つ目は，これらの特性を自分の個性にあった方法で学習し身につける大切さを本人，家族，教師などに伝え，習得方法の例を紹介することとなる。

表6　統合失調症の1次・2次予防実現のための3つのポイント（文献9より引用）

①病前特徴への働きかけ
　対人関係能力
　問題処理技能
　自己評価　など
②ライフイベントに関する心理教育
③2次予防実現のために役立つ情報提供
・精神病理現象（前駆症状，初期症状，精神病体験）の現れ方
・精神科の治療の内容
・早期治療の必要性，有効性
・受診・相談を行う際に利用できる社会資源など

表7　心の病を予防するためのパンフレット―心の健康を守り育てるための9章―（文献9より引用）

①心の病について知っておく利点
②心の病とは？
　―代表的な心の病「統合失調症」のアウトライン―
③統合失調症でよくみられる「空耳」について
④統合失調症でよくみられる「空耳」の内容と影響力
⑤統合失調症でよくみられる「勘繰り」について
⑥統合失調症が起こるきっかけになりやすい生活環境
　―ストレスによるピンチ―
⑦ピンチに陥った時の上手な対応法
　―逆境の受け止め方，しのぎ方―
⑧ピンチに陥らないために役立つこと
　―「転ばぬ先の杖」になりうる事柄―
⑨心の病が出てきた時の対処法と精神科の治療の説明
　―利用できる社会資源の紹介―

表8　予防教育受講者を対象としたアンケート調査の結果（文献9より引用）

質問内容	結果
(1)講義内容への興味・関心の有無	「たいへん興味を持った」または「少し興味を持った」＝88%
(2)パンフレットの内容の理解の可否	「よく理解できる」または「一部理解できる」＝81%（第9項目）～97%（第1項目）
(3)講義内容の有用性の有無	「有用性を感じた」＝80%
(4)講義内容を一般教育の場で扱う必要性	「もっと早く知っておいた方がよい」＝70% 「今頃で（＝大学または専門学校入学時）丁度よい」＝14%

予防の2つ目のポイントは，ライフイベントに関する心理教育である。従来から，統合失調症発症前に様々なライフイベントがみられる場合が多いと知られていたが，筆者らのハイリスク児研究でも同様の結果が得られた。そこで，ライフイベントに関する説明を行い，困難に陥った際の対処法を伝える心理教育が予防に役立つ可能性があると思われる。

3つ目のポイントは，（逆説的であるが）1次予防実現の困難さである。ハイリスク児研究を通して，親の精神状態が比較的安定していて精神科医が日常的に相談に乗っていても，1次予防が容易には実現できないことが明らかになった。そこで，2次予防の重要性があらためてクローズアップされることになる。そしてその実現には，「精神病体験の実際の現れ方や悪影響，精神科の治療の内容と必要性・有効性，相談・治療のために利用できる社会資源」などの情報伝達が役立つ可能性がある。

筆者らは，以上の「3つのポイント」の内容などを一般者向けにわかりやすく解説したパンフレットを作成した[5]。パンフレットの表題は「心の病を予防するためのパンフレット―心の健康を守り育てるための9章―」であり，表7に全9章の表題を示した。

筆者らは，このパンフレットを用いて青年期の一般者を対象とした予防教育を実践し，受講者の関心の度合いや理解の程度などを調べるために，講義終了時に無記名でアンケート調査を行った[6]。表8に結果の概要を示したが，この結果から予防教育がある程度受講者の興味・関心を引いて内容の一部を伝達でき，有用性も一定程度感じとってもらえたとみなせるのではないか，と考えている。

図4　日本版バーチャルハルシネーション用のパンフレット

また，筆者らは統合失調症でよくみられる幻覚症状を疑似体験できる装置「日本版バーチャルハルシネーション（VH）」の精神医学面の監修に携わり，VHの解説パンフレット（図4）を作成した。VHを統合失調症や薬物乱用の予防教育で活

用しうる可能性を考え，試行を始めたところである。

Ⅵ. おわりに

本論では，統合失調症のDUPをめぐる研究結果に触れ，わが国の内外で行われている早期発見・早期治療のためのプロジェクトを紹介した。今後統合失調症の予防をめぐる議論・実践がさらに広がり，統合失調症のDUP短縮が実現し，発症予防に関する臨床研究が進むことが望まれる。

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Evidence of novel neuronal functions of dysbindin, a susceptibility gene for schizophrenia

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Genetic variation in dysbindin (DTNBP1: dystrobrevin-binding protein 1) has recently been shown to be associated with schizophrenia. The dysbindin gene is located at chromosome 6p22.3, one of the most promising susceptibility loci in schizophrenia linkage studies. We attempted to replicate this association in a Japanese sample of 670 patients with schizophrenia and 588 controls. We found a nominally significant association with schizophrenia for four single nucleotide polymorphisms and stronger evidence for association in a multi-marker haplotype analysis ($P = 0.00028$). We then explored functions of dysbindin protein in primary cortical neuronal culture. Overexpression of dysbindin induced the expression of two pre-synaptic proteins, SNAP25 and synapsin I, and increased extracellular basal glutamate levels and release of glutamate evoked by high potassium. Conversely, knockdown of endogenous dysbindin protein by small interfering RNA (siRNA) resulted in the reduction of pre-synaptic protein expression and glutamate release, suggesting that dysbindin might influence exocytotic glutamate release via upregulation of the molecules in pre-synaptic machinery. The overexpression of dysbindin increased phosphorylation of Akt protein and protected cortical neurons against neuronal death due to serum deprivation and these effects were blocked by LY294002, a phosphatidylinositol 3-kinase (PI3-kinase) inhibitor. SiRNA-mediated silencing of dysbindin protein diminished Akt phosphorylation and facilitated neuronal death induced by serum deprivation, suggesting that dysbindin promotes neuronal viability through PI3-kinase-Akt signaling. Genetic variants associated with impairments of these functions of dysbindin could play an important role in the pathogenesis of schizophrenia.

INTRODUCTION

Schizophrenia is a complex genetic disorder characterized by profound disturbances of cognition, emotion and social functioning. It affects ~1% of the general population worldwide. Chromosome 6p is one of the most consistently replicated susceptibility regions in linkage studies of schizophrenia (1). A recent study implicated a gene on chromosome 6p, dysbindin (DTNBP1: dystrobrevin-binding protein 1), as a susceptibility locus in the Irish pedigrees (2). Since then, four studies have reported evidence supporting the association between genetic variants in dysbindin and schizophrenia in

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German, Chinese, Swedish and Irish populations (3–6), while one study failed to replicate positive association in an Irish case–control design (7). In the present study, we attempted to perform an independent association study in a Japanese population of schizophrenic cases and controls.

The pathophysiology of schizophrenia is still unclear; however, this disease is believed to involve genetic abnormalities in developmental processes leading to abnormal synaptic plasticity, including glutamatergic transmission (8,9). Several genes, e.g. dysbindin, neuregulin 1, G72, D-aminoacid oxidase, the regulator of G-protein signaling-4, GRM3 and PPP3CC are described as susceptibility genes for schizophrenia, and those genes may have convergent effects on glutamatergic synapses (10,11). Neuregulin affects the expression and plasticity of the *N*-methyl-D-aspartate (NMDA) receptor (12,13). D-aminoacid oxidase metabolizes D-serine, an endogenous modulator of the NMDA receptor (14), and G72 is probably an activator of D-aminoacid oxidase (15). The regulator of G-protein signaling-4 is the negative regulator of G-protein-coupled receptors, including metabotropic glutamate receptors (16). GRM3 encodes the mGlu3 receptor gene. PPP3CC, the calcineurin γ-subunit, is critical for certain types of NMDA-mediated plasticity. However, no evidence of a role in glutamatergic transmission has been imputed to dysbindin, although dysbindin is believed to play a role in synaptic plasticity and signal transduction. Although dysbindin has recently been cloned as a dystrobrevin-binding protein in mouse (17), little is known about the functions in neurons. Here, we examined neuronal functions of dysbindin and found two novel actions: (1) increased glutamate release with upregulation of pre-synaptic proteins and (2) neurotrophic effect through Akt signaling pathway.

RESULTS

Genetic association analysis

We genotyped six single nucleotide polymorphisms (SNPs) in dysbindin in 670 schizophrenic patients and 588 controls in a Japanese population. The genotype distributions of the six SNPs for the schizophrenic patients and the control subjects were in Hardy–Weinberg equilibrium (data not shown). Allele frequencies of the six SNPs among the patients and controls are shown in Table 1. A significant difference in allele frequency was observed between cases and controls for four SNPs, but not for the remaining two SNPs (Table 1). The G allele of P1635 was in excess in our cases when compared with controls ($\chi^2 = 10.3$, df = 1, $P = 0.0013$, odds ratio = 2.71, 95% CI 1.46–5.79, corrected $P = 0.0078$).

To further analyze the haplotype structure in our sample, we computed the linkage disequilibrium (LD) between the SNPs using D'. D' values ranged between 0.5 and 1.0 and indicated strong to intermediate LD between the markers. Thus, adjacent combinations of up to six markers were examined for association with schizophrenia. Global and individual *P*-values corresponding to haplotypes consisting of adjacent markers and estimated haplotype frequencies in patients and controls are shown in Table 2. All haplotype combinations were significantly associated with schizophrenia, except the P1320–P1763 haplotype. Given this result, we tested the contribution of individual haplotypes to the global result. The G–G haplotype (P1635–P1325), including the G allele of P1635, which was significantly more frequent in our cases (Table 2), was enriched in patients with schizophrenia when compared with controls (estimated frequencies: patients 3.0% versus controls 0.9%, *P*-value = 0.00028, corrected $P = 0.0042$).

Functional analysis in dysbindin-overexpressing cultured neurons

To clarify the function of dysbindin in the central nervous system, we focused on the pre-synaptic machinery in neuronal transmission, as dysbindin is primarily expressed in axonal terminals of the mouse brain (17). Pre-synaptic machinery for exocytotic transmitter release is composed of membrane proteins, cytoskeletal proteins and synaptic vesicle proteins (18). SNAP25 (25 kDa synaptosomal associated protein) and syntaxin are membrane proteins implicated in the docking, priming and fusion of the vesicles. Synapsin I is a cytoskeletal protein associated with the synaptic vesicles in the reserve pool. Synaptotagmin is a synaptic vesicle protein, which has been identified as a calcium sensor protein. Thus, we examined the expression of these synaptic associated molecules after overexpression of dysbindin with virus-mediated gene transfer system. Infected neuronal cultures were doubly stained with GFP signal and immunostaining signal by anti-MAP2 (a neuronal dendritic marker) antibody (Fig. 1A). Approximately 80% of MAP2-positive cells in either control (GFP-infected) or dysbindin-overexpressing (dysbindin- and GFP-infected) cultures were GFP-positive, indicating that the majority of neurons were infected. As shown in Figure 1B, SNAP25 and synapsin I expression tended to be upregulated in dysbindin-overexpressing cultures compared with control (49 and 57%, respectively), whereas the changes of synaptotagmin and syntaxin expression were not observed (data not shown). The levels of class III β-tubulin (TUJ1, a neuronal marker) were not altered in the three conditions (Fig. 1B). We confirmed the overexpression of dysbindin (~17-fold when compared with control) in dysbindin-infected cultures and the expression of GFP in both control and dysbindin-overexpressing cultures (Fig. 1B).

Upregulation of synapsin I and SNAP25 raised the possibility that release of neurotransmitter might be increased by the overexpression of dysbindin. Therefore, we measured the release of glutamate, which is the principle neurotransmitter in these neurons. As expected, the amount of basal glutamate from dysbindin-infected cortical cultures was significantly increased when compared with the uninfected or control cultures (Fig. 1C), indicating that dysbindin overexpression resulted in an elevation of extracellular glutamate. Furthermore, high KCl (HK^+)-evoked exocytotic release of glutamate was enhanced in dysbindin-infected cultures. These results suggest that dysbindin might be one of the regulator proteins in the excitatory neurotransmission.

We then investigated the effects of dysbindin on neuronal viability. Interestingly, it was found that the phosphorylation of Akt, a molecule in the phosphatidylinositol 3-kinase (PI3-kinase) pathway, was significantly enhanced by 67% in the dysbindin-overexpressing cultures, whereas total Akt protein levels were unchanged (Fig. 2A). As the activation of Akt is

Table 1. Allele frequencies of six dysbindin SNPs between the patients with schizophrenia and controls

Marker name	dbSNP ID	Polymorphism major/minor	Location	Minor allele frequency		*P*-value	Odds ratio (95% CI)
				Controls	Patients		
P1655	rs2619539	G/C	Int 5	0.311	0.317	0.748	1.03 (0.87–1.22)
P1635	rs3213207	A/G	Int 4	0.011	0.030	0.0013	2.71 (1.46–5.79)
P1325	rs1011313	G/A	Int 4	0.153	0.166	0.372	0.91 (0.72–1.15)
P1320	rs760761	C/T	Int 3	0.071	0.095	0.027	1.38 (1.04–1.83)
P1763	rs2619522	T/G	Int 1	0.070	0.095	0.022	1.40 (1.05–1.86)
SNPA	rs2619538	T/A	Promoter	0.024	0.040	0.025	1.69 (1.05–2.86)

Table 2. Estimated haplotype frequencies and case–control haplotype results

Markers	*P*-value		Haplotype	Haplotype frequency	
	Global	Individual		Controls	Patients
P1655–P1635	0.0026	0.0003	G–G	0.011	0.030
P1635–P1325	0.00041	0.00028	G–G	0.009	0.030
P1325–P1320	0.0074	0.013	G–T	0.069	0.096
P1320–P1763	0.06	0.02	C–T	0.929	0.904
P1763–SNPA	0.025	0.0047	G–A	0.009	0.025
P1655–P1635–P1325	0.0055	0.001	G–G–G	0.011	0.030
P1635–P1325–P1320	0.0006	0.0009	G–G–T	0.010	0.027
P1325–P1320–P1763	0.027	0.029	G–T–G	0.068	0.095
P1320–P1763–SNPA	0.05	0.0045	T–G–A	0.009	0.025
P1655–P1635–P1325–P1320	0.011	0.0038	G–G–G–T	0.011	0.027
P1635–P1325–P1320–P1763	0.0015	0.001	G–G–T–G	0.010	0.027
P1325–P1320–P1763–SNPA	0.015	0.0019	G–T–G–A	0.007	0.025
P1655–P1635–P1325–P1320–P1763	0.025	0.0028	G–G–G–T–G	0.011	0.027
P1635–P1325–P1320–P1763–SNPA	0.003	0.0016	G–G–T–G–A	0.009	0.026
P1655–P1635–P1325–P1320–P1763–SNPA	0.024	0.0012	G–G–G–T–G–A	0.010	0.026

Case–control haplotype analysis were performed using the permutation method to obtain empirical *P*-values. Global *P*-values and individual *P*-values (lowest *P*-values among the haplotypes) are indicated. Estimated frequency for the haplotype with significant association in controls and patients were shown.

regulated by phosphorylation, overexpression of dysbindin resulted in the activation of Akt. LY294002, a PI3-kinase inhibitor, completely blocked the activation of Akt by the dysbindin overexpression, with no alteration of the expression levels of Akt and TUJ1 proteins (Fig. 2A). As the PI3-kinase pathway is involved in neuronal function and survival (19), we examined the viability of cortical neurons with our virus infection system (Fig. 2B). The overexpression of dysbindin protein itself did not alter neuronal viability when compared with control. However, dysbindin overexpression significantly blocked the reduced viability of cortical cultures by serum deprivation. Additionally, LY294002 significantly inhibited the protective effects of dysbindin, suggesting that the PI3-kinase pathway was involved in the dysbindin-dependent viability promoting effects.

Knockdown analysis of endogenous dysbindin in cultured neurons

We further examined the endogenous dysbindin function in cortical cultures using small interfering RNA (siRNA) for dysbindin. Previously, we reported siRNA-dependent down-regulation of endogenous protein expression in primary cultured neurons (20). Here, we performed transfection of siRNA for dysbindin and confirmed the robust decrease (83%) of endogenous dysbindin protein (Fig. 3A). The protein expression levels of SNAP25 and synapsin I and the phosphorylation level of Akt protein was significantly suppressed after dysbindin-siRNA transfection (43, 37 and 52% of reduction, respectively), although the expression levels of TUJ1 and Akt proteins were not altered (Fig. 3A). Thus, we investigated dysbindin function on glutamate release and neuronal viability under this condition. The amount of basal and released glutamate from dysbindin-siRNA-transfected cortical cultures significantly decreased when compared with the control (scramble) cultures (Fig. 3B), indicating that endogenous dysbindin protein plays a role in the excitatory neurotransmission. The neuronal viability was not changed by dysbindin-siRNA transfection in the presence of horse serum (Fig. 3C). However, dysbindin-siRNA transfection significantly facilitated neuronal death when horse serum was deprived (Fig. 3C), suggesting that the endogenous dysbindin protein has a promoting effect on survival.

DISCUSSION

In the present study, we report a significant association between genetic variation of dysbindin and schizophrenia in a Japanese population. In previous studies, highly significant

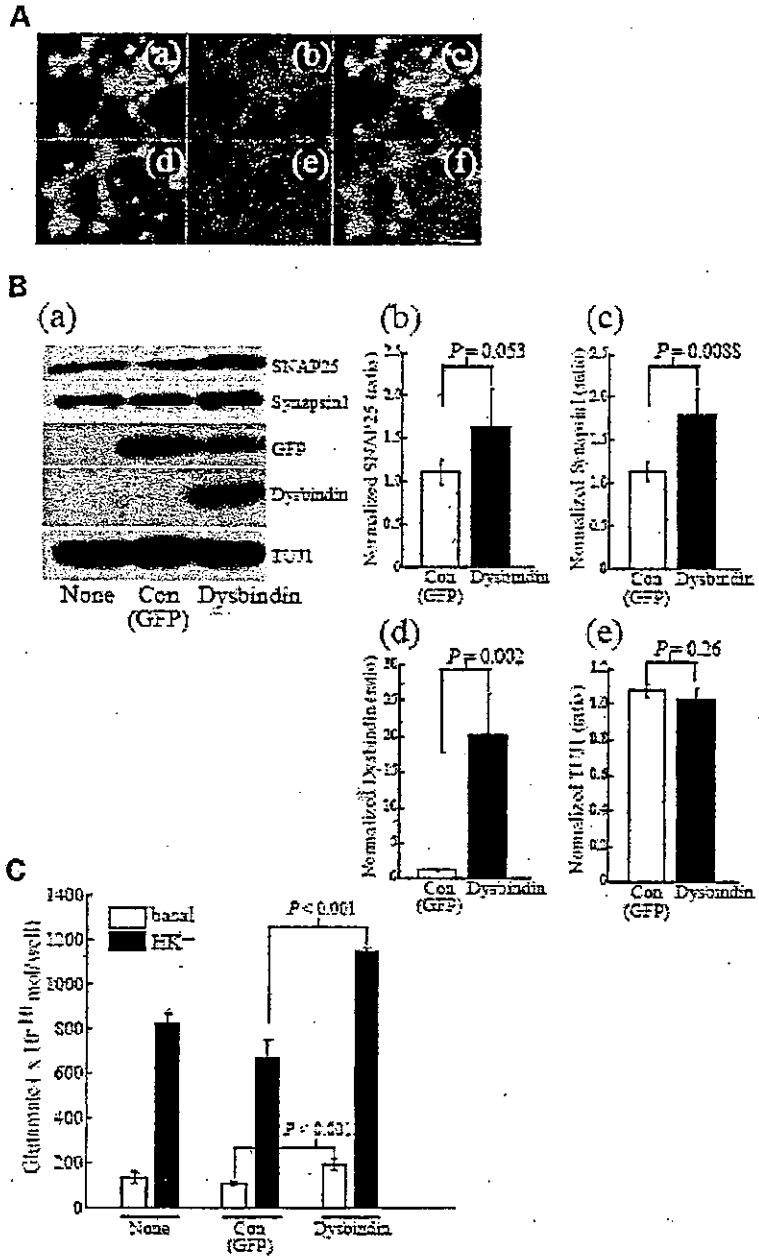

Figure 1. Dysbindin increases the expression of pre-synaptic proteins and glutamate release. (**A**) Double-staining of GFP and MAP2. Cortical cultures (6 days *in vitro*, DIV6) were prepared with viral infection of GFP only (a–c) or with viral infection of GFP and dysbindin (d–f) at DIV4. Images were obtained with GFP (a, d; green) and with immunostaining of anti-MAP2 antibody (b, e; red). Merged images (c, f; yellow) were also shown. (**B**) (a) Upregulation of pre-synaptic proteins. Cortical cultures (DIV6) were prepared without viral infection (None), with viral infection of GFP (Con) or with viral infection of GFP and dysbindin (Dysbindin) at DIV4. The cell lysates were collected at DIV6 and SNAP25, synapsin I, GFP, dysbindin and TUJ1 were detected by western blotting. The immunoblots shown are representative of four independent experiments. (b–e) Quantification of the immunoreactivity of SNAP25, Synapsin I, dysbindin and TUJ1. Data represent mean ± SD of the immunoreactivity from four independent experiments. (**C**) Increase of the released glutamate in dysbindin-overexpressing cortical cultures. Cortical cultures were prepared without viral infection (None), with viral infection of GFP (Con) or with viral infection of GFP and dysbindin (Dysbindin) at DIV4. Basal or HK^+ (50 mM KCl)-evoked release of glutamate was measured at DIV6 (after 48 h from infection). Data represent mean ± SD ($n = 4$).

associations were found for SNPs in introns 4–6, which is consistent with our results. The G allele of P1635, which was significantly in excess in our cases (3.0%), was also over-transmitted in Irish samples (10.2%) (2), whereas this allele was under-transmitted in German samples (17.6%) (3), suggesting that this SNP might be a marker rather than a polymorphism responsible for giving susceptibility. Notably, a high-risk haplotype in our samples was the G–G–T–G

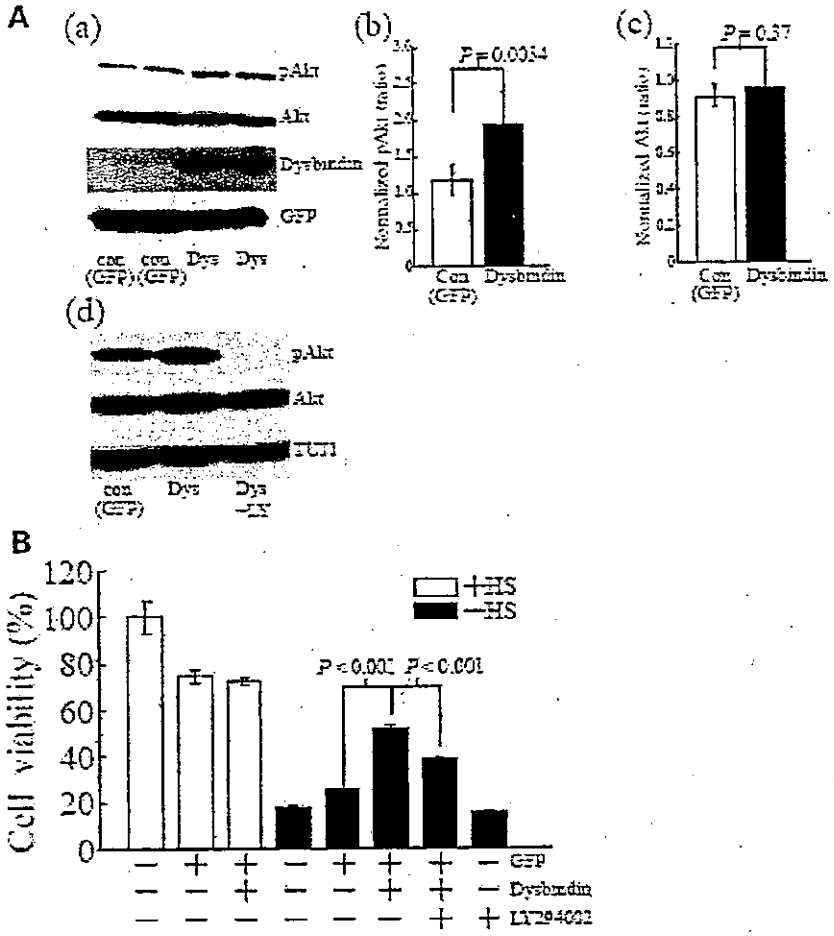

Figure 2. Dysbindin protects cortical neurons through PI3-kinase-Akt signaling. (A) (a) The activation of PI3-kinase pathway in dysbindin-overexpressing cultures. Cortical cultures after DIV4 were treated with viral infection of GFP (Con) or with viral infection of GFP and dysbindin (Dys) for 48 h. (b, c) Quantification of the immunoreactivity of pAkt and total Akt proteins. Data represent mean ± SD of the immunoreactivity from four independent experiments. (d) The inhibitory effect of LY294002 on activation of Akt. Cortical cultures at DIV4 were treated with viral infection of GFP (Con), with viral infection of GFP and dysbindin (Dys) or with viral infection of GFP and dysbindin in the presence of LY294002 (1.0 μM) (Dys + LY) for 48 h. Cortical cultures were harvested at DIV6 for western blotting for pAkt, Akt, dysbindin, GFP or TUJ1. The immunoblots shown are representative of four independent experiments. (B) Neuroprotective effects of dysbindin against serum deprivation. Cortical cultures after DIV4 were treated with viral infection of GFP (Con), with viral infection of GFP and dysbindin (Dysbindin) or with LY294002 (1.0 μM) for 48 h. Deprivation of horse serum (HS) at DIV5 24 h after viral infection is indicated as −HS. Cell viability was determined using the MTT assay at DIV6 48 h after the viral infection and/or 24 h after HS deprivation. Data represent mean ± SD ($n = 8$).

haplotype (P1635–P1325–P1320–P1763), which includes the high-risk haplotype (G–G–G–G–T–G–C–C; P1635–P1325–P1765–P1757–P1320–P1763–P1578–P1792) reported in an Irish sample (6). The frequency of our high-risk haplotype (2.7% in cases versus 1.0% in controls) is lower than that in an Irish population (6%). Novel schizophrenia risk and protective haplotypes (C–A–T, C–A–A, G–G–T; P1655–P1635–SNPA) were recently identified in Cardiff and Dublin samples (21). We also analyzed these haplotypes in our sample and obtained evidence for a significant association with a different haplotype (global P-value = 0.0086, individual P-value = 0.005; G–G–A). Furthermore, the estimated frequencies of C–A–A and G–G–T haplotypes in our sample were <0.1%, although the overall frequencies in Cardiff and Dublin were 33 and 1.4%, respectively. We failed to find a significant association for the C–A–T haplotype (overall frequency, Cardiff and Dublin versus ours, C–A–T: 18 versus 32%). These differences of the haplotype frequencies might be based on the different ethnicity. A false-positive association owing to population stratification could not be excluded in our case–control study, despite the precaution of ethnic matching of this study.

It is of interest to study how genetic variation affects dysbindin function/expression. We do not know that any of the SNPs in our haplotypes are functional. Very little is known about the potential function of specific intronic sequences with regard to protein binding, stability and splicing efficacy. A recent study showed the functional possibility of intronic SNPs on gene expression. For example, an intronic SNP affects the transcriptional efficiency of SLC22A4 *in vitro*, owing to an allelic difference in affinity to Runt-related transcription factor 1, and this SNP is associated with rheumatoid arthritis, one of

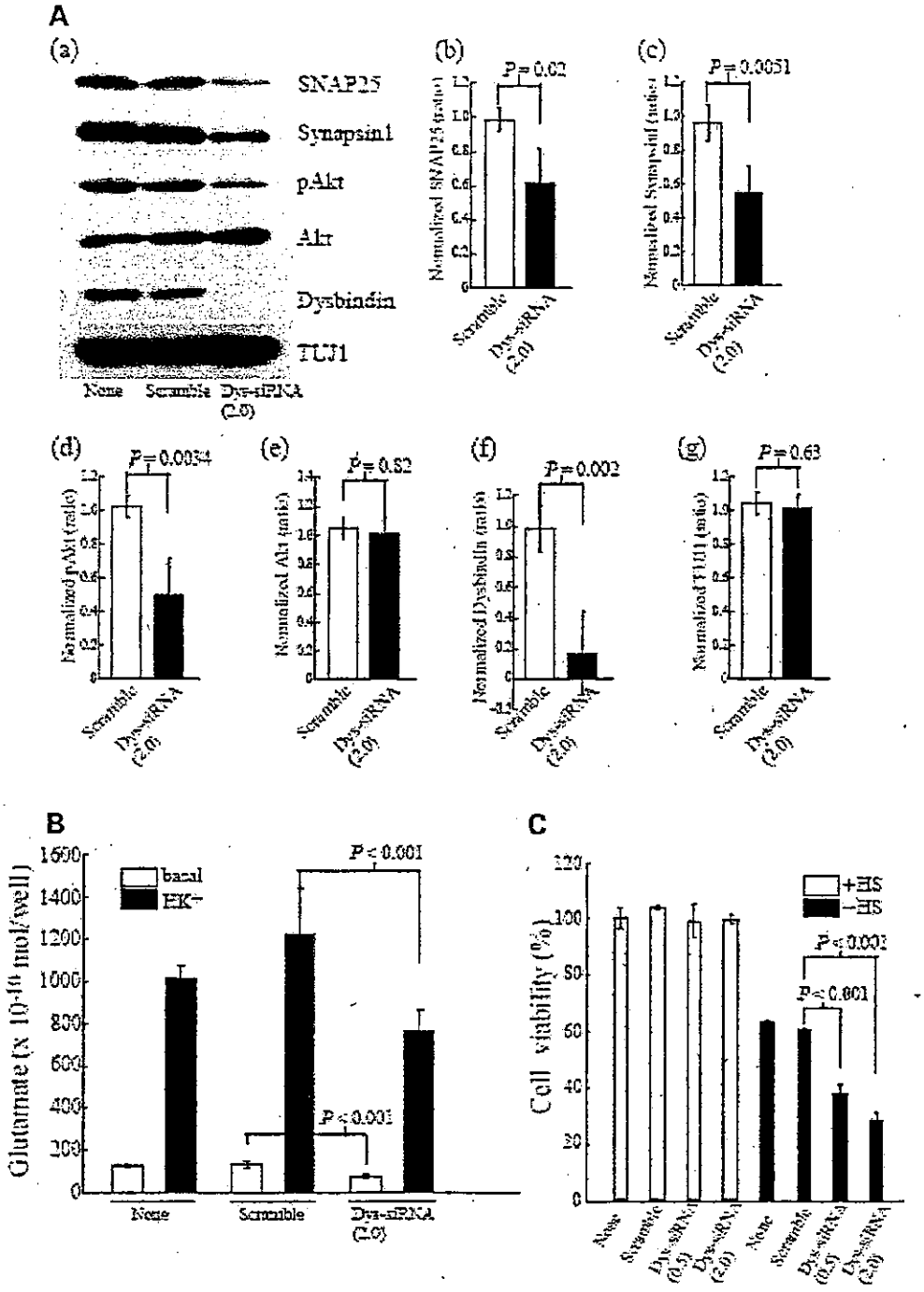

Figure 3. siRNA inhibition of endogenous dysbindin protein modulates protein expression, glutamate release and cell viability. (**A**) (a) Suppression of the pre-synaptic proteins and the phosphorylation of Akt in dysbindin-siRNA-transfected cultures. Cortical cultures after DIV4 were treated with siRNA for dysbindin (dys-siRNA; 2 mg/ml) or control (scramble; 2 mg/ml) for 72 h. Cortical cultures were harvested at DIV7 for western blotting for SNAP25, Synapsin I, pAkt, Akt, dysbindin or TUJ1. The immunoblots shown are representative of four independent experiments. (b–g) Quantification of the immunoreactivity of SNAP25, synapsin I, pAkt, total Akt, dysbindin and TUJ1. Data represent mean ± SD of the immunoreactivity from four independent experiments. (**B**) The reduced glutamate release in dysbindin-siRNA-transfected cultures. Cortical cultures were prepared without transfection (None), with transfection of control siRNA (Scramble; 2 mg/ml) or with transfection of siRNA for dysbindin (dys-siRNA; 2 mg/ml) at DIV4. Basal or HK^+ (50 mM KCl)-evoked release of glutamate was measured at DIV7 (after 72 h from transfection). Data represent the mean ± SD ($n = 6$). (**C**) Facilitation of neuronal death after serum deprivation by dysbindin-siRNA transfection. Cortical cultures after DIV4 were treated without transfection (None), with transfection of control siRNA (Scramble; 0.5 or 2 mg/ml) or with transfection of siRNA for dysbindin (dys-siRNA; 0.5 or 2 mg/ml) for 72 h. Deprivation of horse serum (HS) at DIV6 48 h after transfection is indicated as −HS. Cell viability was determined using the MTT assay at DIV7 72 h after the transfection and/or 24 h after HS deprivation. Data represent mean ± SD ($n = 8$).

the complex genetic diseases like schizophrenia (22). Alternatively, an unknown functional polymorphism, which is in LD with the SNPs and/or haplotypes, may be responsible for providing susceptibility to schizophrenia.

To date, association of dysbindin with schizophrenia has been confirmed across diverse populations. In addition, decreased expression of dysbindin mRNA and protein levels has been observed in prefrontal cortex and hippocampus of postmortem brain in schizophrenic patients (23–25). As dysbindin is distributed at least in part in axonal terminals (17), we focused on the possible role of dysbindin in neuronal transmission. We used two techniques, overexpression and knockdown, to investigate neuronal function of dysbindin. As the overexpression levels of dysbindin using sindbis virus were quite high when compared with the control level (~17-fold), the results could have non-physiological effects. However, the results from the knockdown experiments of the endogenous dysbindin protein were consistent with those from overexpression experiments. Our experiments suggest that dysbindin regulates the expression of SNAP25 and synapsin I proteins in the pre-synaptic machinery and is associated with increased glutamate release. SNAP25 is one of the fundamental molecular components of the SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors) protein complex, which is involved in intracellular vesicle trafficking and neurotransmitter release (18). Synapsin I is localized to the synaptic vesicles that are both docked and located away from the plasma membrane (18). Reduction of SNAP25 protein has been observed in frontal cortex of schizophrenia patients (26) and synapsin I protein was found to be reduced in the hippocampus of patients with schizophrenia (27). Hypofunction of glutamatergic system has been implicated in the neuropathology in schizophrenia (8). The abuse of phencyclidine, an NMDA receptor antagonist, results in positive symptoms, negative symptoms and cognitive impairments, similar to schizophrenic patients. The postmortem brain studies suggested impaired glutamatergic systems, e.g. reduced glutamate level, decreased AMPA receptor binding and expression and reduced NMDA receptor expression in several brain areas, including frontal cortex and hippocampus.

Our experiments also suggest the survival effect of dysbindin protein on cortical neurons against serum deprivation through the PI3-kinase-Akt signaling pathway. Thus, dysbindin might play an important role in neuronal vulnerability. Impaired PI3-kinase-Akt signaling in schizophrenia has been reported recently (28). Dysbindin expression in the brain of schizophrenic patients was reduced (23–25) and our data suggested that the downregulation of dysbindin expression suppressed the phosphorylation levels of Akt. Taken together, impaired PI3-kinase-Akt signaling in the schizophrenic brain might be due, in part, to the decreased expression of dysbindin. As dysbindin may affect neuronal viability through Akt activation, dysbindin-Akt signaling might be involved in early disruptions producing long-term vulnerability that leads to the onset of schizophrenia symptoms. As PI3-kinase-Akt signaling is activated by several growth factors such as brain-derived neurotrophic factor, nerve growth factor and insulin-like growth factors through tyrosine kinase receptors (19), the regulation of this system might be associated with dysbindin.

The Hermansky–Pudlak syndrome defines a group of autosomal recessive disorders characterized by deficiencies in lysosome-related organelles complex-1 (BLOC-1). Hermansky–Pudlak type-7 is caused by a nonsense mutation of dysbindin, which is a component of the BLOC-1 (29). Biological roles of BLOC-1 are still unknown; however, it might be involved in vesicle docking and fusion. Sandy mouse, which has a deleted dysbindin gene, expresses no dysbindin (29). Thus, this mouse could be a powerful tool for investigating brain function of dysbindin *in vivo*. It is of interest to examine the pre-synaptic protein expression, glutamate release, Akt phosphorylation and neuronal vulnerability *in vivo* using this mouse.

We have demonstrated the additional support for the genetic association between dysbindin and schizophrenia in a relatively large sample and the evidence of novel functions of dysbindin in cultured neurons. Our results suggest that an abnormality of dysbindin might influence glutamatergic systems and Akt signaling. Further investigation is necessary to elucidate the mechanisms of Akt activation and upregulation of pre-synaptic molecules by dysbindin.

MATERIALS AND METHODS

Subjects

Subjects for the association study were 670 patients with schizophrenia [males: 50.6%, mean age of 44.2 years (SD 14.6)] and 588 healthy comparison subjects [males: 48.7%, mean age of 36.2 years (SD 12.4)]. All the subjects were biologically unrelated Japanese patients. Consensus diagnosis was made for each patient by at least two psychiatrists according to the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) criteria. Control subjects were healthy volunteers who had no current or past contact to psychiatric services. After description of the study, written informed consent was obtained from every subject. The study protocol was approved by institutional ethical committees (Fujita Health University School of Medicine, Showa University School of Medicine and National Center of Neurology and Psychiatry).

SNP genotyping

Venous blood was drawn from the subjects and genomic DNA was extracted from whole blood according to the standard procedures. Six SNPs (P1655, P1635, P1325, P1320, P1763 and SNPA) adopted in the work of Straub *et al.* (2) and Williams *et al.* (21) were genotyped using the TaqMan 5′-exonuclease allelic discrimination assay, described previously (30,31). Briefly, the probes and primers for detection of the SNP were as follows. P1655: forward primer 5′-AGTTTTTAT CACTAATCAAAATGAAACAGCCTTT-3′, reverse primer 5′-CTCATTCTGTTATAACTAGTCTGACATGGT-3′, probe 1 5′-VIC-TATTAGCTATGATAGTGTTTTAT-MGB-3′ and probe 2 5′-FAM-ATTAGCTATGATAGTCTTTTAT-MGB -3′; P1635: forward primer 5′-GGAACTTTTCTTTGAAGA CTTCCTTTCG-3′, reverse primer 5′-ACCACTAACAACC AAAAAGAAAACAAACA-3′, probe 1 5′-VIC-TAAAGCC AATAATTACC-MGB-3′ and probe 2 5′-FAM-AGCCAG

TAATTACC-MGB-3′; P1325: forward primer 5′-GATATG ACTCCTTAATTCACAGGCTACAG-3′, reverse primer 5′-GTTACTGCACACAAGCAACTGTTAA-3′, probe 1 5′-VIC -AATGGATGTTGCATTAGT-MGB-3′ and probe 2 5′-FAM -ATGGATGTTGCGTTAGT-MGB-3′; P1320: forward primer 5′-CCAATCCATTCTTTTATTGACATGGAGTTT-3′, reverse primer 5′-TGATTTTGACCAAGTCCATTGTGTCT -3′, probe 1 5′-VIC-AAAAGCACAAACAACAAG-MGB-3′ and probe 2 5′-FAM-AAAAGCACAAATAACAAG-MGB-3′; P1763: forward primer 5′-GGCAGAAGCAGTGAGTGAGA-3′, reverse primer 5′-TGGGCTCTTATGTCTACCTTTCCTAAA -3′, probe 1 5′-VIC-TCACCTGGATGTCAGC-MGB-3′ and probe 2 5′-FAM-ACCTGGCTGTCAGC-MGB-3′; SNPA: forward primer 5′-TCTGTTATGTGCCATTCACTGTTTT-3′, reverse primer 5′-TAGGGCTGGGATTGGATGA-3′, probe 1 5′-VIC-AGCAGTTTACATCTGGG-MGB-3′ and probe 2 5′-FAM-AGCAGTTTACATCAGGG-MGB-3′. PCR cycling conditions were 95°C for 10 min, 45 cycles of 92°C for 15 s and 60°C for 1 min.

Cell culture

Dissociated cortical cultures were prepared from postnatal 2- or 3-day-old rat (SLC, Shizuoka, Japan) cortex, as described previously (32,33). Briefly, cells were gently dissociated with a plastic pipette after digestion with papain (90 U/ml, Sigma) at 37°C. The dissociated cells were plated at a final density of 5×10^5 per cm^2 on polyethyleneimine-coated 12- or 24-well plates (4 and 2 cm^2 surface area/well, respectively; Corning, NY, USA) or cover glasses (Matsunami, Osaka, Japan) attached to flexiperm (VIVASCIENCE, Gottingen, Germany). The culture medium consisted of 5% precolostrum newborn calf serum, 5% heated-inactivated horse serum and 90% of a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 medium containing 15 mM HEPES buffer, pH 7.4, 30 nM Na_2SeO_3 and 1.9 mg/ml of $NaHCO_3$.

Sindbis virus

A bicistronic vector plasmid (pSinEGdsp) was provided by Dr Kawamura (Niigata University, Japan). The plasmid was derived from pSinRep5 (Invitrogen, USA) and had two subgenomic promoters followed by a multiple cloning site for arbitrary gene insertion and an EGFP open reading frame, thus the virus can produce arbitrary protein and EGFP independently in the infected cell, as previously described (34). Dysbindin cDNAs amplified by RT–PCR with specific primer pairs (forward 5′-ACGCGTCAATGCTGGAGACCCTTCG-3′ and reverse 5′-GCATGCCAATTTAAGAGTCGCTGTCC-3′) were inserted at the *Mlu*1 and *Sph*1 sites of the plasmid. Each plasmid was cleaved with *Pac*1, and used as a template for mRNA transcription *in vitro* using mMESSAGE mMACHINE kit (Ambion, USA). Pseudovirions were produced according to the experimental procedure of Invitrogen. Baby hamster kidney (BHK) cells were transfected with each mRNA and 26S helper mRNA (Invitrogen) by electroporation (1250 V/cm, 50 μF, single pulse) using Gene Pulser2 (BioRad, USA). The cells were incubated with DMEM supplemented with 10% FCS for 24 h at 37°C, the supernatants were collected as pseudovirion-containing solutions.

Immunocytochemistry

Cultured neurons were fixed with 4% paraformaldehyde for 20 min and then rinsed three times with PBS. Subsequently, cultured cells were permeabilized with 0.2% Triton X-100 in PBS for 5 min at room temperature. The primary antibodies (anti-MAP2; Sigma) with 3% skim milk in PBS were applied overnight at 4°C. After washing, cells were incubated with secondary antibodies (Alexa Fluor, Molecular Probes) for 1 h at room temperature. Fluorescent images were captured by an inverted microscope (Axiovert 200, Zeiss) with a CCD (cool SNAPfx) purchased from Zeiss. Monochrome images were turned into color and analyzed using software (Slide BookTM 3.0, Intelligent Imaging Innovations, Inc., Denver, CO, USA). The images of GFP were analyzed with the same software.

Immunoblotting

Cells were lysed in SDS lysis buffer containing 1% SDS, 20 mM Tris–HCl (pH 7.4), 5 mM EDTA (pH 8.0), 10 mM NaF, 2 mM Na_3VO_4, 0.5 mM phenylarsine oxide and 1 mM phenylmethylsulfonyl fluoride. Lysates were centrifuged at 15 000 rpm for 60 min at 4°C, and the supernatants were collected for analysis. Samples were heat denatured with the standard SDS sample buffer. Immunoblottings were carried out as described previously (35). Briefly, immunoblottings were carried out with anti-SNAP25 antibody (1:3000, mouse monoclonal, Synaptic System, Gottingen, Germany), anti-synapsin I antibody (1:1000, rabbit anti-serum, Chemicon), anti-synaptotagmin antibody (1:1000, mouse monoclonal, BD Transduction Laboratory), anti-syntaxin antibody (1:3000, mouse monoclonal, Sigma), anti-GFP antibody (1:1000, rabbit polyclonal, MBL, Nagoya, Japan), anti-dysbindin antibody (23) (1:100, rabbit polyclonal), anti-TUJ1 antibody (1:5000, mouse monoclonal, Berkeley antibody company, CA, USA), anti-Akt antibody (1:1000, rabbit anti-serum, Cell Signaling) and anti-phospho-Akt antibody (Ser473, 1:1000, rabbit anti-serum, Cell Signaling) in TBS containing 1% non-fat dried milk. The immunoblotting experiments were performed four times and they were quantitatively analyzed by capturing images on films using a scanner (Epson, Tokyo, Japan) in conjunction with the Lane and Spot Analyzer software (version 6.0, ATTO, Tokyo, Japan).

Anti-dysbindin antibody was produced as described previously (36). Briefly, the peptide synthesized (QSDEEEVQVDTALC: 320–333 amino acid residue of human dysbindin, with no homology in any mammalian protein) was conjugated with maleimide-activated keyhole limpet hemocyanin and immunized to two rabbits. The titer was measured by ELISA and sera of high titer against the peptide were obtained from both rabbits. The sera were affinity purified by a column conjugated with the immunized peptide.

Detection of glutamate release

The amount of glutamate released from the cultures was measured as previously reported (33,35). The glutamate released into the modified HEPES-buffered Krebs–Ringer assay buffer (KRH; 130 mM NaCl, 5 mM KCl, 1.2 mM

NaH_2PO_4, 1.8 mM $CaCl_2$, 10 mM glucose, 1% bovine serum albumin and 25 mM HEPES, pH 7.4) were measured by HPLC (Shimadzu, Kyoto, Japan) with a fluorescence detector (excitation wavelength, 340 nm; emission wavelength, 445 nm, Shimadzu). For stimulation of cortical neurons, we used a HK^+ KRH solution consisting of 85 mM NaCl, 50 mM KCl, 1.2 mM NaH_2PO_4, 1.8 mM $CaCl_2$, 10 mM glucose, 1% bovine serum albumin and 25 mM HEPES, pH 7.4. Before exposing the cultures to HK^+ solution (1 min), basal fractions were collected. The glutamate release experiments were performed three times with independent cultures to confirm reproducibility.

MTT assay

To examine the cell viability, the metabolic activity of mitochondria was estimated by measuring the mitochondrial-dependent conversion of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma). We performed the viral infection or transfection of siRNA and then, the serum was deprived from culture medium. MTT (0.5 mg/ml in PBS) was added to each well at 24 h after serum deprivation. MTT was incubated for 1.5 h at 37°C. Then, the medium was carefully aspirated, and 200 µl of acidified isopropyl alcohol was added to solubilize the colored formazan product. Absorbance was determined at 550 nm on a scanning multi-well plate reader (Bio-Rad) after agitating the plates for 5 min on a shaker.

siRNA transfection

We used 23 nt siRNA duplexes with two 3′ overhanging nucleotides targeting position 182–204 (aagugacaagucaaga gaagcaa) of human dysbindin mRNA. Scrambled sequence (aacgaugagaacgaucaagaaga), which had no homology to any mammalian mRNA, was used as a control siRNA. Both sense and antisense strands were synthesized by Dharmacon Research Inc (Lafayette, PA, USA). SiRNA duplexes in the 2′-ACE deprotected and desalted form were dissolved in a 1× universal buffer (Dharmacon Research Inc). Transfection of both siRNAs was performed using NeuroPORTER™ (Gene Therapy Systems, Inc., San Diego, CA, USA), as reported (20).

Statistical analysis

Statistical analysis of association studies was performed using SNPAlyse (DYNACOM, Yokohama, Japan). The presence of Hardy–Weinberg equilibrium was examined by using the χ^2-test for goodness of fit. Allele distributions between patients and controls were analyzed by the χ^2-test for independence. The measure of LD, denoted as D', was calculated from the haplotype frequency using the expectation–maximization algorithm. Case–control haplotype analysis was performed by the permutation method to obtain the empirical significance (37). The global P-values represent the overall significance using the χ^2-test when the observed versus expected frequencies of all the haplotypes are considered together. The individual haplotypes were tested for association by grouping all others together and applying the χ^2-test with 1 df. P-values were calculated on the basis of 10 000 replications. Statistical analysis of neurobiological assays was performed by Students' t-test. All P-values reported are two tailed. Statistical significance was defined at $P < 0.05$. To be conservative, Bonferroni corrections were applied for multiple comparisons, e.g. number of analyzed SNPs and haplotypes, although SNPs were in LD.

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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.
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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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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.

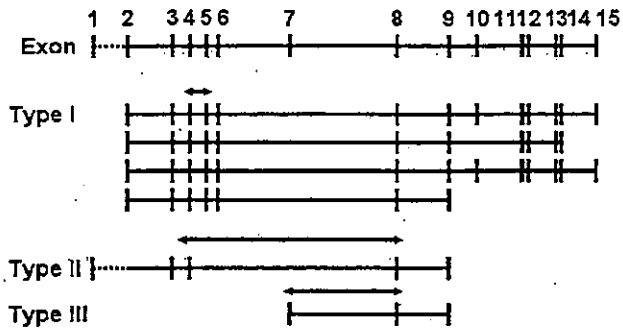

Figure 1 NRG-1 structure and probe design. Human NRG-1 mRNA has 15 exons. The exon usages of type I, type II or type III isoforms of NRG-1 are shown. Arrows indicate the location of primers and probes specific for type I, type II or type III isoforms of NRG-1.

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-

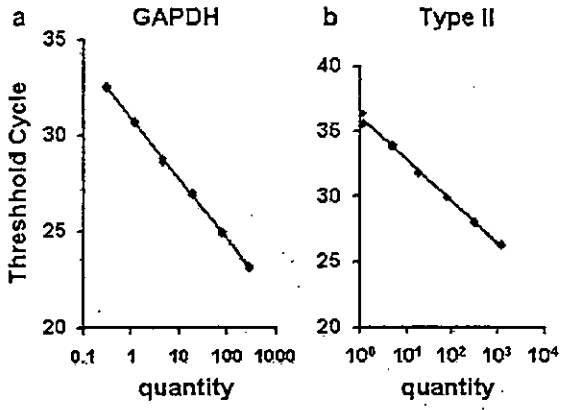

Figure 2 Standard curves for control (housekeeping genes) and NRG-1 type II. Standard curves for GAPDH (a) and type II (b). The quantity represents an amount of cDNA prepared from 1 ng of total RNA in the PCR reaction. R^2 values are 1.000 and 0.995 for GAPDH and type II.

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,

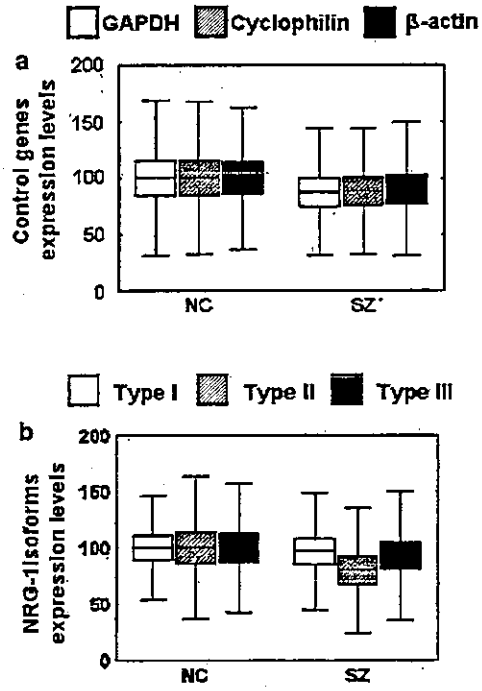

Figure 3 mRNA expression levels of NRG-1 isoforms. The expression levels of housekeeping genes (a) and NRG-1 isoforms (b) were measured in the DLPFC of normal control subjects (NC) and patients with schizophrenia (SZ). The expression levels were calculated by comparison to the percentage of average of 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) ±33.8 (SD) *vs* schizophrenia patients: 109.1±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).

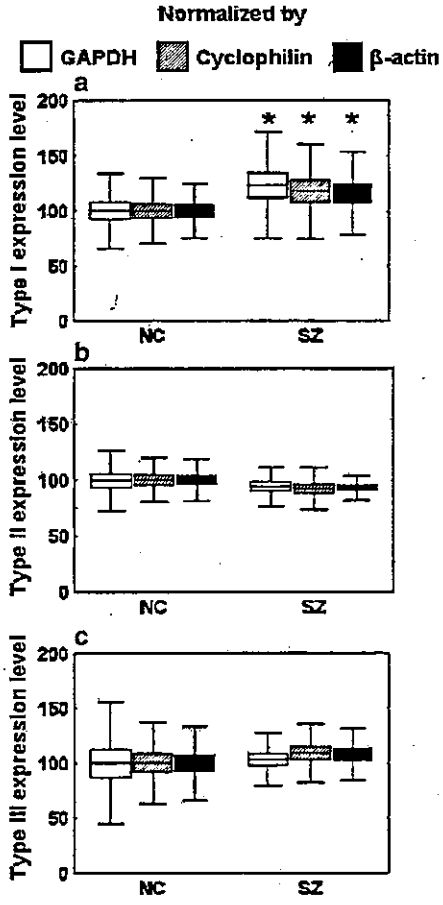

Figure 4 Relative expression levels of NRG-1 type I (a), type II (b), and type III (c) isoforms normalized by GAPDH, cyclophilin or β-actin in the DLPFC of normal control subjects (NC) and patients with schizophrenia (SZ). Significant group differences by ANCOVA are indicated by $^*P<0.05$.

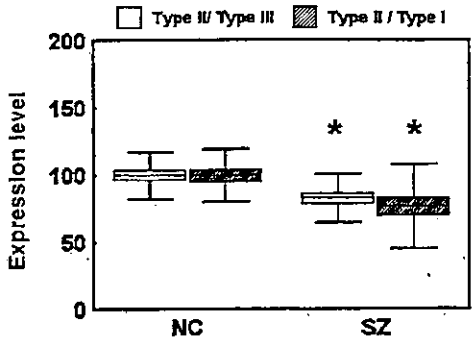

Figure 5 Relative expression ratios of Type II normalized by Type I or Type III in DLPFC of normal control subjects (NC) and patients with schizophrenia (SZ). $^*P < 0.05$.

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±37.6 (SD), while two carriers (N=12) had 99.2±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